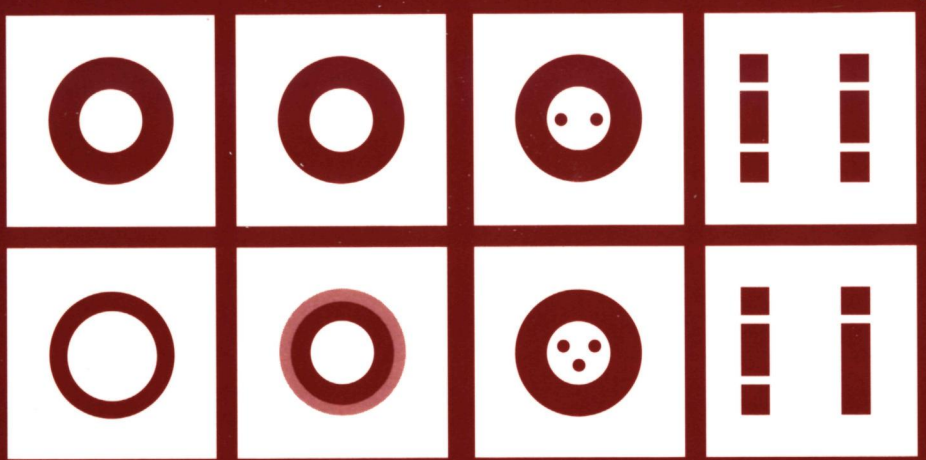
# Molecular and morphological approaches in diagnostic melanocytic pathology



Peter E.J. de Wit

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de Medische Wetenschappen

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CONTENTS

# CHAPTER 1

# General introduction

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Pathology of malignant melanoma, including new markers and techniques in diagnosis and prognosis

## INTRODUCTION

The melanin pigment in the human skin that protects it from the adverse effect of sunlight is produced by specialized neural crest derived cells: melanocytes. These cells localize predominantly in the basal epidermal cell layer and in the hair follicles. Normally the perikaryon of melanocytes are separated from each other by about three to ten keratinocytes (68). When benign melanocytic cells proliferate and form clusters or nests, these cells have been termed naevus cells and the lesions are called "melanocytic naevi" or "naevocellular naevi". Naevi may be regarded as benign neoplasms of melanocytes. Malignant neoplasms of melanocytic cells are termed "malignant melanoma", synonymously used with "melanoma" (42,46,81,99).

Two principle pathways of tumorigenesis in melanoma have been proposed: it is thought that there may be a de novo proliferation of epidermal melanocytes which turned malignant, whereas also a more gradual progression can occur via specific melanocytic naevi as an intermediate stage. Tumor progression is the concept of the evolution from the normal cell to the metastatic phenotype through a series of discrete stages with in each further step an additional growth advantage (66,98,109). Because cutaneous melanocytic lesions are readily visible and accessible, this concept has been extensively studied in melanoma. With respect to clinical features and to architectural and cytological characteristics at the histological level, an intermediate lesion between the common acquired naevus on the one hand and melanoma on the other hand has been recognized and termed "dysplastic naevus". This lesion is considered a probably clinically relevant, potential precursor of melanoma (45).

### *Melanoma and the pathologist*

Cutaneous malignant melanoma is a highly metastatic tumor, disseminating already at relatively small sizes. In The Netherlands the age adjusted incidence rate per 100.000 is about 8 with a female to male ratio of about 1.5. The age adjusted mortality rate per 100.000 is $\pm$ 2.5. About 2.5% of all cancers detected per year in The Netherlands concern malignant melanomas, mostly men or women in their fourties or fifties. Death due to cancer involves a malignant melanoma in less than 1% (37,69,105,144,144). Although these figures indicate that malignant melanoma is not a "big killer", in relatively young adults it is an important cause of death and next to this, many clinical and basic research activities have focussed on melanoma because of several intriguing aspects. From a clinical point of view, next to the challenge of the clinical diagnosis and related

aspects such as early detection or screening programs, it is one of the few tumors which so far show some promising results in immunotherapeutical trials, whereas sentinel node procedures and isolated limb perfusion protocols form modern surgical therapeutic modalities (10,11,22,74,76,80, 82,90,97,100,112,126,141, 147,150). With respect to basic research, malignant melanoma and its precursors form an attractive model because of the possibility to study molecular aspects of tumor progression through a series of discrete stages which can be identified histologically (19,25,44,64,121,149). For the general pathologist melanocytic lesions form a substantial part of the daily work: many melanocytic lesions are being excised and, since melanoma has many faces with a wide variety in morphology (the Lues under the tumors) and look-a-likes, a pertained careful consideration of every melanocytic lesion is necessary to avoid histopathological misdiagnoses with serious consequences (14,46,81,99,115).

Although at present malignant melanoma is not a frequently encountered tumor, this may change as one of the most worrisome aspects of malignant melanoma is its rapid worldwide rise in incidence. A doubling of incidence every ten to fourteen years has been described (53,89,92,145). Also in The Netherlands data point to a similar increase in incidence, while between 1950 and 1988 also the annual age standardized death rates have increased about four fold, despite the fact that more melanomas are being detected in an earlier stage (37,105,144,145). These trends have concentrated attention to the epidemiology of melanoma and to possible etiological factors which can explain the increasing incidence (20,57,62,65,72,86,94,104,118,143). Solar and non-solar factors have been put forward and recommendations were made, aiming at primary prevention (avoidance of too much uv-light) (7,8,38,51). The underlying research data and discussions have also led to an increased awareness amongst physicians and in the general population, of the fact that when a cutaneous malignant melanoma is detected and excised in an early stage, the prognosis is excellent (secondary prevention stimulated through campaigns) (18,76,77,93,103,108,146). Probably due to this increased interest in recognizing melanoma at a curable stage and in recognizing precursor-stages, more pigmented lesions are being removed for histological examination (12,24,58,84,88,95,113). So, pathologists nowadays are presented with many melanocytic lesions for diagnosis, especially with a high proportion of lesions that are diagnostically challenging.

From a practical point of view the pathologist, when dealing with a melanocytic lesion, has to answer three main questions: 1) Is the lesion malignant and if so,

what is the prognosis? 2) If benign, is the lesion a marker of an increased risk for (developing) a melanoma at another site? 3) Is the lesion completely excised and with what margin?
The unequivocal identification of a malignant melanoma, the determination of the free margin and the assessment of prognostic factors are important because of their clinical consequences with respect to re-excision margins, sentinel node and lymph node dissection procedures and follow-up regimens. Inclusion of patients in trials investigating the possible benefits of systemic or regional adjuvant therapy in high risk patients also requires an unequivocal and correct diagnosis (11,80).

Although the correct categorization of most of the benign cutaneous melanocytic lesions does not influence their clinical management, the significance of recognizing the specific benign entity with its clinicopathological characteristics is that recognizing the entity as such helps the pathologist to discriminate the lesion in question from malignant melanoma (26,28,87,134,136,152). Although at careful consideration most melanocytic lesions are readily diagnosed histologically, a significant proportion remains difficult to categorize. As documented in Chapter 2 a substantial part of the problems in this field encountered by pathologists consists of the differential diagnosis between malignant melanoma and dysplastic naevus and between malignant melanoma and Spitz naevus. Furthermore, the correct identification of a dysplastic naevus is important because, like melanoma itself, it may indicate an increased risk of developing a (subsequent) melanoma, possibly in the context of the dysplastic naevus syndrome, the latter potentially also of importance for the family of the patient. Although reexcision of a not completely excised naevus is always advocated to avoid the problem of the occurrence of a subsequent so-called "pseudo-melanoma", the total excision of a dysplastic naevus is mandatory, because of its role as a relevant precursor lesion (45,135).
So, many clinically relevant problems encountered by pathologists in melanocytic pathology are related to melanomas, dysplastic naevi and Spitz naevi. Clinicopathological characteristics of these lesions are discussed below.

***Clinico-pathological characteristics of melanoma***
As stated, melanomas show a wide variety in clinical presentation, histology and biological behaviour and different classification systems for melanoma exist (29,30,48,67,133). One system underlines the distinction between radial (horizon-

tal) growth phase melanomas and vertical growth phase melanomas and is more in line with the concept of tumor progression. Its basis is the concept of the relevance of the histological recognition of those lesions which have metastatic capacity through identification of a vertical growth phase. In contrast, in this concept lesions with only a radial growth phase are considered to lack metastatic potential.

The other widely known (conventional) histogenetic classification system is based on clinico-pathological characteristics in conjunction with epidemiological properties and is more derived from concepts on the etiology of melanoma. Thus, both classification systems seem to have additional value. The most characteristic aspects of both classification systems of melanoma are summarized in Tables I and II.

The histological diagnosis of melanoma depends on the recognition of a constellation of histological features, no single feature being diagnostic of melanoma. Both architectural and cytological characteristics are important (14,46,81,99).

**Table I: Main clinico-histological features in four histogenetic subtypes of melanoma**

| | SSM* | NM | LMM | ALM |
|---|---|---|---|---|
| Age | fourties | fifties | sixties | sixties |
| Race | mainly whites | mainly whites | only whites | all |
| Relative frequency** | 65% | 10% | 10% | 5% |
| Localization | all sites | all sites | mostly head and neck | nailbeds, soles,palms |
| Actinic skin-damage | uncommon | uncommon | present | absent |
| History of sun-exposure | intermittent | intermittent | cumulative | |
| RGP-pattern | pagetoid | not applicable | lentiginous | lentiginous |
| Usual cell type | epithelioid hyperplasia | epithelioid hyperplasia | spindle | spindle |
| Epidermis | common | uncommon | atrophy | marked hyperplasia |
| Desmoplasia | uncommon | uncommon | common | common |
| Neurotropism | uncommon | uncommon | common | common |

* SSM: superficial spreading melanoma; NM: nodular melanoma; LMM: lentigo malignant melanoma; ALM: acro lentiginous melanoma; RGP: radial growth phase

** 10% unclassifiable

**Table II: Radial growth phase versus vertical growth phase invasive melanoma**

| RGP (microinvasive) | VGP |
|---|---|
| - Single cells and small aggregates (usually <5-10) of melanoma cells of similar size invading in papillary dermis (no growth preference) | - Single cohesive cellular nodule or plaque or multiple nests (usually >15-25 cells in diameter) in papillary dermis (growth preference) |
| - Dermal nests are no larger than intraepidermal nests, and do not distort the surrounding tissues | - Individual cellular nests are larger than nests in RGP, and distort the surrounding tissues |
| - Cells are similar to the intraepidermal melanoma cells | - Dermal melanoma cells differ from those in the RGP |
| - Mitoses are rare in dermal part | - Mitoses common in dermal part |
| - Usually prominent lymphocytic infiltrate | - Lymphocytic infiltrate less prominent |
| - Almost always thinner than 1 mm | - Often thicker than 1 mm |

Architectural characteristics include asymmetry of the mainly relatively large (> 6mm) lesion with a poor circumscription of the melanocytic proliferation and variation in size and shape of the nests. Further, ascent of atypical cells is an important hallmark: upward migration of atypical melanocytes in a random pattern, with single cells or groups of a few cells reaching the granular layer (pagetoid growth). Lack of so-called maturation is mostly found. Cytological atypia (present in the majority of cells) include nuclear changes with nuclear enlargement, pleomorphism, hyperchromasia and one or more prominent nucleoli and also cytoplasmic changes often including abundant eosinophilic cytoplasma in epithelioid cells and so-called dust-like melanin. Mitoses are usually found, also in the deeper parts of the lesion where there is an expanding pattern without respect for surrounding tissues. Also atypical mitoses and necrosis may be found.

The histological differential diagnosis of malignant melanoma versus other clinico-pathological entities includes a large number of benign and malignant tumors both of melanocytic and non-melanocytic origin (Table III). In most cases where non-melanocytic lesions are considered in the differential diagnosis, the non-melanocytic character can be identified by careful attention to histological details in routinely stained sections. Histochemistry and immunohistochemistry can be helpful (36,130).
With respect to the differential diagnosis of the conventional subtypes of

**Table III: Differential diagnosis of malignant melanoma***

**Superficial spreading melanoma**
- Melanocytic tumors
  - Lentigo malignant melanoma
  - Dysplastic melanocytic naevus
  - Halo naevus
  - Spindle and epithelioid cell naevus
  - Pigmented spindle cell naevus
  - Recurrent melanocytic naevus
  - Other melanocytic naevi with prominent pagetoid spread
- Nonmelanocytic tumors
  - Squamous cell carcinoma
  - Paget's disease, mammary or extramammary
  - Sebaceous carcinoma
  - Cutaneous T-cell lymphoma
  - Epidermotropic eccrine carcinoma
  - Metastatic carcinoma, other
  - Angiosarcoma
  - Kaposi's sarcoma
  - Leiomyosarcoma
  - Atypical fibroxanthoma
  - Malignant fibrous histiocytoma

**Nodular melanoma**
- Melanocytic tumors
  - Metastatic melanoma
  - Spindle and epithelioid cell naevus
  - Pigmented spindle cell naevus
  - Cellular blue naevus
- Nonmelanocytic tumors
  - Squamous cell carcinoma
  - Atypical fibroxanthoma
  - Malignant fibrous histiocytoma
  - Cellular neurothekeoma
  - Malignant peripheral nerve sheath tumors
  - Angiosarcoma

**Nodular melanoma (continued)**
- Kaposi's sarcoma
- Histiocytoid hemangioma
- Leiomyosarcoma

**Lentigo malignant melanoma**
- Melanocytic tumors
  - Solar lentigo
  - Dysplastic naevus
  - Pigmented spindle cell naevus
  - Superficial spreading melanoma
- Nonmelanocytic tumors
  - Squamous cell carcinoma, spindle-cell type
  - Atypical fibroxanthoma
  - Cellular neurothekeoma
  - Malignant peripheral nerve sheath tumor

**Acrolentiginous melanoma**
- Melanocytic tumors
  - Melanocytic naevi of acral skin
  - Lentiginous melanocytic proliferations of acral skin
  - Pigmented spindle cell naevus
- Nonmelanocytic tumors
  - Squamous cell carcinoma, spindle-cell type
  - Atypical fibroxanthoma
  - Cellular neurothekeoma
  - Malignant peripheral nerve sheath tumor
  - Angiosarcoma
  - Kaposi's sarcoma
  - Histiocytoid hemangioma
  - Leiomyosarcoma

*After Barnhill and Mihm, reference 14, page 54

melanoma, it can be difficult to discriminate one subtype from another, and it is accepted that about 10% of melanomas is not further classifiable. Although important from a clinico-epidemiological point of view and also for the diagnostic work of the pathologist because the different conventional subtypes have in part their specific benign look-a-likes, the specific subtype of melanoma does not

seem to be of prognostic significance. Based on a vast literature, prognostic factors are clinical and pathological tumor stage, site of the primairy lesion, age and sex of the patient, tumor thickness measured according to Breslow, level of invasion according to Clark, presence and extent of ulceration, mitotic rate and presence of satellites. Of these the Breslow-thickness (in millimeters) is the most important and robust prognostic factor in localised disease. The 5 year survival percentage is more than 95% in very thin (<0.76 mm) melanomas whereas in thick melanomas ($\geq$ 4 mm) this drops to around 50%. Melanomas of an intermediate thickness show survival rates in between (23,39,52,85,113).
So, as most thin (mostly radial growth phase) melanomas will not metastasize, the correct classification of a melanocytic lesion as either benign or malignant is especially important with increasing thickness because the prognosis and clinical management of a thick melanoma on the one hand or of a thick naevus at the other hand is totally different. As stated, in the experience of many pathologists two specific differential diagnostic problems are relatively frequently encountered i.e. melanoma versus dysplastic naevus and melanoma versus Spitz naevus.

***The dysplastic naevus***

Histologically, a melanocytic naevus is a focal area of increased melanocytic cellularity with nested growth of mostly non-atypical melanocytes. Clinically, except for cosmetic significance, naevi are important only for their relation to melanoma. Different forms of naevi have been described with more or less specific clinical and histopathological characteristics. Most of these naevi are only important for the pathologist because the various subtypes should be recognized as the specific look-a-likes of the various types of malignant melanoma, since melanoma is known for its wide variation in histomorphological presentation (16, 26,87,134,138,152).
The significance of dysplastic naevi can be summarized as being clinically relevant potential precursors of melanoma, being markers of an increased risk for melanoma and being simulants of melanoma (41,45). Potential precursors are lesions in which the rate of cancer development is greater than that found in normal tissue. The clinical importance is that by excision of melanocytic precursors death from melanoma (which would have evolved if the lesion was left alone) can be prevented (120). For most types of naevi neither a clinical nor a histopathological role as a precursor of melanoma or as a marker of an increased risk for melanoma is known at present. For giant congenital naevi however, an increased risk of the occurrence of a melanoma in the naevus is known and

surgical excision is therefore advocated (91,142). Also malignant change at the site of an acquired naevus is a clinically and histologically documented phenomenon (47). If at microscopic examination a melanoma is found together with remnants of a benign melanocytic naevus, its histological aspect almost always is that of a common acquired naevus or that of a dysplastic naevus. The estimation of the frequency of progression through naevi to melanoma has been based mainly on such histological evidence of naevus remnants in melanoma. Between 5% and 39% (median 22%) of melanomas exhibit a dysplastic naevus remnant whereas a part of a common acquired naevus is reported in an additional 10% to 21% (median 15%) of cases (45).

Based on the histomorphology of naevus-remnants in melanomas and following the concept of tumor progression, all common acquired naevi in the general population may be potential very early precursors of melanoma. It is not justifiable and clearly impractical to remove all these naevi because everybody of Caucasian complexion has about 10-30 such lesions and the annual incidence of melanoma for instance in The Netherlands is about 8 per 100.000. Calculated in a crude theoretical model approximately 2% of common acquired naevi may progress to dysplastic naevi (131). The dysplastic naevus was described as a subtype of naevus carrying a higher, possibly clinical significant risk of malignant transformation to melanoma.

In the late seventies melanoma kindreds were reported with many, clinically atypical naevi that were common among family members (Table IV). These clinical atypical naevi where referred to as B-K-moles (after the initials of the index-families), later as large atypical naevi whereas in 1980 it was suggested that the atypical moles should be called dysplastic naevi (31,32,55,56,60,71,83, 119). The atypical moles as well as the melanomas were considered to be part of a syndrome. It was referred to as familial atypical multiple mole melanoma (FAMMM) syndrome and later also as dysplastic naevus syndrome (DNS) (83,102). Lesions similar to dysplastic naevi in the familial hereditary dysplastic naevus syndrome were also reported in non-familial (sporadic) melanoma patients (sporadic dysplastic naevus syndrome) (43,106). Persons with the dysplastic naevus syndrome (either familial or sporadic) have a highly increased risk of developing a melanoma, so that their identification is of clinical importance (78,79). Further studies demonstrated that dysplastic naevi occurred also in individuals who neither had melanoma, nor were members of hereditary melanoma kindreds (9,61,65). Now, clinically atypical naevi are considered not uncommon, there reported incidence ranging from 5% to 20% (median 13%)

**Table IV: Dysplastic naevus, clinical characteristics**

- 5 mm or more in largest diameter
- Macular, sometimes with papular central part
- Asymmetrical shape
- Irregular border
- Ill-defined border
- Irregular pigmentation
- Red hue

(45). The occurrence of clinically atypical naevi is associated with an increased risk for melanoma for persons who are not members of hereditary melanoma kindreds also after adjustment for the total number of naevi on the body and for other risk factors. An increasing risk for melanoma has been described for an increasing number of total naevi and also for an increasing number of clinically atypical naevi (128). Cohort studies indicate that the recognition of clinically atypical naevi permits the identification of high risk groups, especially individuals who have a personal or family history of melanoma, large numbers of atypical lesions or a complex phenotype of multiple risk factors. In high risk patients, melanomas can be detected in an earlier stage (27,50,54,55,95,123,124,146). So, the detection of a high risk phenotype depends primairly on (family)history and physical examination (45). Although therefore preferably prior to the excision of a suspect melanocytic lesion a total skin examination and a specific family history should always be obtained, in our experience this is not always done and possibly impractical in current clinical practice. Here is a role for the histopathologist who, next to excluding melanoma, by reporting melanocytic dysplasia may trigger the search for clinical risk factors associated with an increased melanoma risk.

Few studies have attempted to evaluate the epidemiological significance with respect to melanoma risk of just dysplasia as determined histologically. Although it is not yet known whether or not independent of clinical atypia, dysplasia seems to be associated with a higher melanoma risk (9). An emerging question is how many naevi of what (histological) type in connection with which other factors determine a significant increased risk (13,15). This question is somewhat artificial since both on clinical and histological grounds as well as emerging from the concept of tumor progression there probably is a biological continuum

between common acquired naevi without atypia towards dysplastic naevi with evident architectural and cytological atypia. This continuum probably also feeds the discussion on the criteria needed for a histological diagnosis of dysplastic naevus, and in line with this the basic discussion about the reproducible recognition of the lesion. Also the bare existence of a clinicopathological entity defined by clinical atypia and histological dysplasia has been questioned because the borders defining the lesion are under discussion (1,2,3,4). To solve the problem, such discussions on matters at the interpretation and defenition level seem only part of the solution (33,34,35,40,49,63,75,101,107,116,117,122,125,127,129, 132,137,140). Because the scoring of the individual features forms the basic material from which the pathologists starts to interpret, it seems more important to see whether the features contributing to "dysplasia", can be recognized reproducibly. Subsequently further studies can determine whether, to what extent and how these features are associated with increased melanoma risk, whether they are independent of clinical atypia and whether the association is stronger for one or more (specific combinations of) features. From there, boundaries of parts of the spectrum can be proposed arbitrarily based on clinical pathological correlation and clinical relevance: as also melanoma risk so far seems to be a continuum related to the "naevus-phenotype" the discussion tends towards the question what relative risk and thus what degree of atypia/dysplasia is taken as the boundary from which level upwards physicians and health authorities consider it worth of taking patients into surveillance programs. Based on these considerations, it was felt worthwhile to study and describe the reproducibility of the histological features generally scored in melanocytic lesions, with emphasis on dysplastic naevi (Chapter 3).

The dysplastic naevus as a high-grade simulant of melanoma is a known histopathological problem. In this differential diagnosis, next to morphological features, other phenotypical and genotypical characteristics may be of value, although also here the gradual accumulation of "markers" along the line of tumor progression blurs the boundaries: that is, no single marker will problably exactly discern between two histologically defined neighbouring stages of disease along a line of tumor progression. Although such a problem is frequently encountered in the differential diagnosis between a dysplastic naevus and a thin radial growth phase melanoma, the biological and clinical significance of these lesions is stated to be similar as they are both regarded as lesions without capacity for metastasis. The clinically more relevant problem is the differentiation between the deeper

**Table V: Naevus cells versus small melanoma cells in the deeper parts of melanomas**

| Naevus cells of preexisting naevus | Deeply invading small melanoma cells |
|---|---|
| - No mitoses | - Mitoses sometimes present |
| - Small non-atypical nuclei | - Larger atypical nuclei |
| - No nucleoli at base | - Prominent nucleoli |
| - Single cells between collagen at base | - Nests and fascicles rather than single cells |
| - Lymphocytes absent | - Lymphocytes present |
| - Little or no pigment | - Irregularly scattered pigmented cells at base of lesion; dusty pigment |
| - Small uniform nests at base | - Larger variable nests at base |

dermal part of a malignant melanoma versus the naevus remnant underlying a micro invasive, radial growth phase melanoma (Table V). This differentiation has major impact on the Breslow thickness and thus on prognosis and clinical management. As a basis towards an application in this differential diagnosis, we studied the epidermal growth factor receptor as a possible progression-marker (chapter 4 of this thesis).

*Spitz naevus*

The name "Spitz naevus" pays tribute to Sophie Spitz who in 1948 first described histological criteria to distinguish the Spitz naevus from malignant melanoma (6,139). When the term "Spitz naevus" is used, it refers to a group of melanocytic naevi, most often acquired, composed of large epithelioid and/or spindle cells (21,73,96,110,111,114,148,149). Another frequently used name is spindle and epithelioid cell naevus. Most Spitz naevi are diagnosed in Caucasian children and young adults, often involving the legs and the face although the lesion may occur anywhere on the body. Although the Spitz naevus is typically a circumscribed, non-pigmented, asymptomatic, dome-shaped nodule less than 1 cm in diameter, there is a large variation in the clinical presentation. A correct diagnosis on clinical parameters alone is very difficult. On the other hand the clinical features of Spitz naevus, especially the age of the patient and the body-site of occurrence should always be taken into account together with the histological appearance to reach a final diagnosis.

**Table VI: Histological features commonly found in typical Spitz naevi***

- Hyperplasia of the epidermis
- Overall symmetry
- Lateral borders sharp
- Retraction spaces between intraepidermal nests of melanocytes and adjacent epidermis
- Ascending melanocytes absent or rare
- Kamino bodies present, often confluent
- Regular architecture of dermal component
- Infiltrating margins at base non-disruptive
- Maturation with increasing depth
- Mitoses in superficial parts, but very rare in deep parts

* After Mooi and Krausz, reference 99, page 171.

Histologically, most Spitz naevi are compound lesions, whereas approximately 10% are solely junctional and a further 15% purely intradermal. The lesion consists typically of a proliferation of large epithelioid and/or spindle-shaped melanocytes (Table VI). The epithelioid melanocytes are mostly oval or polygonal cells with distinct borders. The cytoplasm is often abundant and stains mostly homogeneous eosinophilic. The nuclei of epithelioid cells are large and centrally located. The chromatin is usually finely dispersed. Typically there is a distinct, single round large nucleolus, centrally located in the nucleus. Multi-nucleated or bi-nucleated cells are commonly observed. The spindle-shaped melanocytes are large and often rather plump in appearance. The chromatin of the usually oval nuclei is finely stippled. Distinct nucleoli are typical.

The described features can be encountered in typical Spitz naevi. However, Spitz naevi may also show features more generally associated with melanoma. Thus, architectural disorder and cytological atypia may be encountered which is insufficient for a diagnosis of melanoma but also not consistent with a diagnosis of typical Spitz naevus (16,17,136,152). This reflects the experience of most pathologists that based on the discussed criteria one can confidently distinguish on histological grounds between a typical Spitz naevus and a typical melanoma but that difficult cases are encountered regularly. Here, as a first step a scrupulous scoring of the clinical and histological parameters can be of help to reach a

**Table VII: Spitz naevus versus nodular melanoma**

| Spitz naevus | Nodular melanoma |
|---|---|
| - Symmetrical | - Asymmetrical |
| - Epidermal hyperplasia | - Epidermal hyperplasia uncommon |
| - Ovoid nests of cells, perpendicular to epidermis | - Nests variable in size, shape, and orientation |
| - Pagetoid spread rare | - Pagetoid spread common |
| - Uniform nests at base | - Larger nests at base (different size) |
| - Kamino bodies usually present | - Kamino bodies rare |
| - Mitoses absent or rare and very rare at base | - Mitotic rate often high, mitoses common at base |
| - Cellular symmetry/uniformity | - Asymmetry |
| - Maturation with descent to base | - Maturation rare |
| - Single cells between collagen at base | - Fascicles/nests in the reticular dermis at base |

correct diagnosis (26,28,70,138). Important differential diagnostic features to be scored are listed in Table VII. A second step may be to ask for the opinion of a colleague, more experienced in this particular field of pathology. A further approach to minimize the number of "impossible cases" may be the application of additional techniques. To this end, we performed DNA-in situ hybridization and allelotyping experiments (chapters 5-7 of this thesis).

## OUTLINE OF THIS THESIS

This thesis deals with several aspects of specific differential diagnostic problems, regularly encountered in the field of melanocytic pathology.

Chapter 2 documents the character and relative extent of difficult areas in melanocytic pathology encountered in general practice as deducted from the cases submitted to the Pathology Panel of the Dutch Melanoma Working Group, the bimonthly meetings of which the author of this thesis has been attending for the past seven years. The results give guidance to the focussing on the problems regarding the dysplastic naevus and the Spitz naevus: histomorphological, as well as immunohistochemical, DNA-in situ hybridization and allelotyping approaches

were used.

Chapter 3 describes the attempt made, through an interobserver study among experts in the field of melanocytic pathology in Europe, to come up with a set of best reproducible histomorphological parameters in melanocytic lesions leading to the highest chance of obtaining a reproducible, although still arbitrary histological "boundary" between the group of dysplastic naevi and common naevocellular naevi.

Chapter 4 presents a study on the expression of the epidermal growth factor receptor on melanocytic cell lines and tissues at the protein and mRNA level and its use as a possible progression marker.

In chapter 5 a study on the feasibility of the application of the DNA-in situ hybridization technique on melanocytic cell lines and tissues is described, especially on tissue sections from routinely processed, formalin-fixed, paraffin-embedded tissues.

Chapter 6 describes a study on the possible implementation of the DNA-in situ hybridization technique in the histological differential diagnosis between Spitz naevus and malignant melanoma.

In chapter 7 a pilot study is described in which routinely processed tissues were used as starting material to perform allelotyping experiments on Spitz naevi and malignant melanomas in order to investigate whether the phenomenon of loss of heterozygosity may be used for diagnostic purposes.

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# CHAPTER 2

# Quality assessment by expert opinion in melanoma pathology: experience of the Pathology Panel of the Dutch Melanoma Working Party

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**ABSTRACT**

Some cutaneous melanocytic lesions are notoriously difficult to diagnose by histopathology. For that reason the Pathology Panel of the Dutch Melanoma Working Party was instituted and is regularly approached to provide an expert opinion on problem cases. In order to identify the most common diagnostic problems, 1069 consecutive referral cases of submitted lesions (1992 to 1994 inclusive) were analysed.

About 60% of the requests came from small laboratories, with up to three consultant pathologists. Two-thirds of the lesions reviewed concerned women and nearly 50% of the patients were 30 years of age or younger.

In 8% of the cases the referring pathologists felt unable to make a confident diagnosis, in 14% melanoma was suspected and in 12% a differential diagnosis only had been formulated. The panel felt able to provide an unequivocal diagnosis in 93% of the requests.

Of the 158 lesions classified as invasive melanoma by the referring pathologists, 22 were considered to be benign by the panel. Conversely, 108 invasive melanomas (panel diagnosis) had originally been considered as benign lesions, dysplastic naevus or melanoma in situ.

These high numbers of discordancies reflect the intrinsic difficulty of the differential diagnoses in this selected material submitted to the panel. Diagnostic difficulties were most often encountered with Spitz naevi and dysplastic naevi.

Although the rate of overdiagnosis and underdiagnosis is quite high, in the majority the diagnosis of the referring pathologist matched the diagnosis of the panel. This may reflect a proper awareness of difficult melanocytic lesions in pathology practice.

The activities of the Pathology Panel of the Dutch Melanoma Working Party contribute to the improvement of the quality of diagnosis in cutaneous melanocytic lesions as they increase the number of unequivocal diagnoses and reduce the number of incorrect diagnoses. On the basis of the systematic comparison of the diagnosis by the referring pathologist and the panel, postgraduate teaching and quality control can be more focused on specific diagnostic problems. *(J Pathol 1997; in press).*

## INTRODUCTION

Quality assurance is of great importance in the diagnosis and treatment of cancer patients. This is underscored by the current policy of various international organizations which stimulate quality control projects in different aspects of the management of cancer patients. As an example, in the field of cutaneous melanoma, quality control within the framework of the EORTC (European Organization for Research and Treatment of Cancer) Malignant Melanoma Cooperative Group, is executed in the context of clinical trials, i.e. by means of site visits by an expert surgeon to centres which have entered patients treated with isolated perfusion therapy (1). With regard to the histological diagnosis and micro-staging of lesions from patients entered in such studies and others, quality assurance is pursued by the reviewing of the slides by expert pathologists.

High quality standards within the framework of international collaboration are not possible without a continuous effort to maintain and improve the care of melanoma patients at the national and regional level. Thus, melanoma working parties are now active in several countries, including The Netherlands. The Dutch Melanoma Working Party was founded in 1986. It consists of a multi-disciplinary forum of the various specialties involved in melanoma management, aiming at improving of the prevention, early diagnosis and treatment of melanoma. Three consensus texts on melanoma were prepared by the group under the guidance of the National Organization for Quality Assurance in Hospitals (2). Members of the working party also act as consultants for colleagues with less experience in melanoma. In addition, the working party provides continuous education in melanoma pathology. In order to be able to make such an effort to be as effective as possible, insight is required gained into the problems encountered by pathologists. This is of importance since it provides subjects for targeted training.

About 300 cases are sent annually to the Pathology Panel of the Dutch Melanoma Working Party for expert opinion. The purpose of this article is to report on a consecutive series of 1069 such cases, with emphasis on the specific diagnostic problems and major discrepancies between the diagnoses of referring pathologists and panel pathologists. Recommendations are made with respect to targeted quality control and continuous education.

## MATERIALS AND METHODS

### *Working methods of the Pathology Panel and materials*

The Pathology Panel of the Dutch Melanoma Party consists of three pathologists

(WJM, DJR and ES). It operates on a consultative basis: pathologists who have difficulties in making an unequivocal diagnosis of a lesion may send representative slides and/or paraffin blocks of the lesion along with a tentative diagnosis to a member of the panel. The referring pathologist receives a written report from the panel member within one week. Where doubt remains, the panel member may send the slides to other panel members. The cases are discussed among the panel members at regular bi-monthly meetings. Just occasionally, this panel discussion leads to a clinically relevant change in the original panel diagnosis which is then promptly communicated in an additional report.
Requests are received from pathologists throughout the country and occasionally from abroad. Each consultation is recorded in the national computerised system on pathological diagnoses (Pathologisch Anatomisch Landelijk Geautomatiseerd Archief, PALGA) which facilitates retrieval of follow-up data. Also from each consultation data are collected by registering items through a standard list: gender, date of birth, site of the lesion, name of the hospital/department of the referring colleague, name of the consulted pathologist, diagnosis of the referring pathologist, available material and panel diagnosis. The panel members use information mentioned in the report and the accompanying letter from the referring pathologist to complete this list.

The diagnoses of the referring pathologists and the panel pathologist(s) are registered as standard entities listed in Table I. For this study, these entities were grouped in the following categories: no diagnosis, common acquired naevus, Spitz naevus, other special type of naevus, dysplastic naevus, melanoma in situ, suspect melanoma, invasive melanoma, and others. The category "differential diagnosis" was also included.

***Methods used for evaluation of data***

Comparison of the referring pathologists' and the panel's diagnoses was made in terms of overdiagnosis and underdiagnosis.
Overdiagnosis was defined from the perspective of clinical relevance, including therapeutic consequences. The following situations were regarded as overdiagnosis: the diagnostic category of the referring pathologist was "suspect melanoma" or "invasive melanoma" while the diagnostic category of the panel was "common acquired naevus", "Spitz naevus", "special type of naevus", "dysplastic naevus" or "melanoma in situ".
Underdiagnosis was defined as the situation in which lesions were classified as "common acquired naevus", "Spitz naevus", "special type of naevus", "dysplastic

**Table I: Categorization of separate diagnoses into diagnostic categories**

| Separate diagnosis | Category |
|---|---|
| No diagnosis | No diagnosis |
| Common acquired naevus | Common acquired naevus |
| Spitz naevus | Spitz naevus |
| Congenital naevus | Other special type of naevus |
| Pigmented spindle cell naevus | Other special type of naevus |
| Blue naevus | Other special type of naevus |
| Combined naevus | Other special type of naevus |
| Dysplastic naevus | Dysplastic naevus |
| Lentigo maligna | Melanoma in situ |
| Melanoma in situ | Melanoma in situ |
| Melanoma in situ with dysplastic naevus | Melanoma in situ |
| Suspicion of malignant melanoma | Suspect melanoma |
| Superficial spreading melanoma | Invasive Melanoma |
| Nodular melanoma | Invasive Melanoma |
| Lentigo maligna melanoma | Invasive Melanoma |
| Acral lentiginous melanoma | Invasive Melanoma |
| Melanoma unclassified | Invasive Melanoma |
| Melanoma in congenital naevus | Invasive Melanoma |
| Melanoma in pre-existent naevus | Invasive Melanoma |
| Melanoma metastasis | Invasive Melanoma |
| Differential diagnosis | Differential diagnosis |
| Other diagnosis | Other |

naevus" or "melanoma in situ" by the referring pathologist and as "suspect melanoma" or "invasive melanoma" by the panel.

## RESULTS

The Pathology Panel examined a total number of 1069 cases over a three year period: 333 in 1992, 359 in 1993, and 377 in 1994.

### *Referring pathologist*

The requests came from pathologists working in hospitals or institutes throughout The Netherlands and from some pathologists abroad (8 cases in 1992, 24 cases in 1993 and 24 cases in 1994). Number of requests per institute varied from 1 to 35 per year. Four institutes sent 15 or more cases per year. The institutes were grouped according to the number of pathologists and categories were made: small institutes

(1-3 pathologists), intermediate (4-6 pathologists), large (>6 pathologists) and unknown. Thirty-five small institutes over the three years period submitted 614 cases (median: 30; range: 1-88), 14 "intermediate" institutes sent in 194 cases (median: 13; range: 2-33) and 10 large institutes sent in 179 cases (median: 12.5; range: 2-61). Thus, small institutes with fewer pathologists sent in more cases.

***Age and gender of the patients***

In Table II the distribution of gender and age of all submitted cases and of the cases with a panel diagnosis of "invasive melanoma" are presented. About two-thirds of all submitted cases concerned women and nearly half of the patients were 30 years of age or younger. The younger the age, especially in children, the more lesions that were submitted, but few invasive melanomas were identified by the panel in childhood cases (Table II). Nevertheless, the percentage of invasive melanomas in young individuals amoung the invasive melanomas diagnosed by the panel was high when compared to the age distribution in the general population. In the panel's series, about one fourth of invasive melanomas were from patients of 30 years or younger while in the Dutch cancer registry about 10% of invasive melanoma patients are under the age of 30 (3). This reflects the particular difficulties encountered by pathologists in diagnosing lesions in especially younger patients, as well, perhaps, a reluctance to diagnose invasive melanoma in a young patient without seeking a second opinion. Of the submitted cases in male patients younger than sixteen years of age 9% (7 of 74) were diagnosed as invasive melanoma whereas this was 67% (12 of 18) in patients over 75 years (Table II).

With regard to gender, the panel diagnosed more cases of invasive melanomas in females than in males as compared to the general population: 34% males and 66% females diagnosed by the panel against 41% and 59% respectively, in the general population (3). This may be due to the overall surplus of lesions from females submitted for consultation.

***Site of the lesion***

The site of the lesion was recorded in 692 cases. The most common sites, in decreasing order of frequency, were: legs 34% (234), trunk 33% (226), head/neck region 17% (115), arms 12% (85) and other sites 5% (32). Other sites included conjunctiva (11), external genitals (10), juxtacutaneous mucosa (5), hip (1), abdominal cavity (3), lumbal spinal column (1) and lymph node (1).

**Table II: Age and gender of the patients**

| age[a] | gender | | | |
|---|---|---|---|---|
| | all cases submitted[b] | | panel diagnosis invasive melanoma[c] | |
| | male | female | male | female |
| 0 -15 | 74 (19%) | 56 (9%) | 7 (6%) | 5 (2%) |
| 16-30 | 101 (26%) | 215 (33%) | 20 (17%) | 49 (21%) |
| 31-45 | 95 (25%) | 201 (31%) | 38 (32%) | 78 (33%) |
| 46-60 | 62 (16%) | 107 (16%) | 28 (24%) | 58 (24%) |
| 61-75 | 36 (9%) | 59 (9%) | 14 (12%) | 37 (16%) |
| 75+ | 18 (5%) | 14 (2%) | 12 (10%) | 10 (4%) |
| unknown | 3 | 6 | 1 | 0 |
| total | 389 (37%) | 658 (63%) | 120 (34%) | 237 (66%) |

[a]: in years
[b]: gender unknown: 22
[c]: gender unknown: 7

Of the 364 lesions diagnosed by the panel as invasive melanoma, the sites were: trunk 35% (83), legs 32% (76), head/neck region 19% (44), arms 10% (24) and other localizations 3% (8). The site was unknown in 129 cases.

The body distribution of the invasive melanomas diagnosed by the panel was compared with the lesions coded in 1993 as invasive melanoma in the national computerised system PALGA. The distribution of the sites of the lesions in PALGA was: legs 43% (98), trunk 30% (67), head/neck region 13% (30) and arms 14% (32). Compared with the distribution seen in the general invasive melanoma population, the panel invasive melanoma cases were somewhat less often localized on the legs and more often in the head/neck region. This may reflect a tendency to seek confirmation of the pathology diagnosis invasive melanoma in the head/neck region since its impact in terms of surgical treatment and cosmetic consequences is greater than at other sites.

*Diagnosis*

Table III presents the diagnoses of the refering pathologist versus the diagnoses of the panel, after categorization. Relatively many of the lesions referred were diagnosed by the panel as Spitz naevus or other special types of naevus. The referring pathologists submitted many lesions with suspicion of malignancy, but few

**Table III: Diagnosis of the referring pathologist versus diagnosis of the Pathology Panel**

| Diagnosis of | Pathology Panel | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| referring pathologist | No diagn.* | Com. naevus | Spitz naevus | Special naevus | Dyspl. naevus | Mel in situ | Suspect mel. | Invasive mel. | Diff. diagn. | Other | Total |
| No diagn.* | **1** | 8 | 21 | 12 | 12 | 3 | 6 | 21 | 2 | 4 | 90 |
| Com. naevus | 0 | **48** | 5 | 12 | 3 | 2 | 1 | 11 | 1 | 1 | 84 |
| Spitz naevus | 0 | 2 | **109** | 11 | 2 | 0 | 8 | 17 | 5 | 3 | 157 |
| Special naevus | 1 | 1 | 3 | **39** | 0 | 1 | 3 | 4 | 2 | 0 | 54 |
| Dyspl. naevus | 1 | 24 | 16 | 20 | **56** | 13 | 9 | 35 | 2 | 0 | 176 |
| Melanoma in situ | 1 | 1 | 1 | 1 | 5 | **16** | 2 | 18 | 1 | 0 | 46 |
| Suspect mel. | 2 | 11 | 25 | 8 | 8 | 3 | **16** | 71 | 4 | 3 | 151 |
| Invasive mel. | 0 | 3 | 10 | 4 | 2 | 3 | 2 | **133** | 1 | 0 | 158 |
| Diff diagn. | 1 | 5 | 34 | 15 | 11 | 4 | 3 | 51 | **5** | 2 | 131 |
| Other | 0 | 0 | 1 | 4 | 0 | 0 | 0 | 3 | 0 | **14** | 22 |
| Total | 7 | 103 | 225 | 126 | 99 | 45 | 50 | 364 | 23 | 27 | 1069 |

* No diagn.: No diagnosis, Com. naevus: Common acquired naevus, Special naevus: Other special type of naevus; Dyspl. naevus: Dysplastic naevus; Mel. in situ: Melanoma in situ; Suspect mel.: Suspect melanoma, Invasive mel.: Invasive melanoma, Diff. diagn.: Differential diagnosis.

lesions with a diagnosis of "melanoma in situ".

From the total number of lesions categorized as "other" by the referring pathologist or the panel (22+27), a substantial number were classified as non-melanocytic soft tissue tumour (20), melanocytic lesion of the conjunctiva (8), or melanocytic tumour not specified in the other mentioned categories (8). A final group of 13 lesions consisted of various other diagnoses such as "scar" and "dermatitis".
The total number of lesions in which the panel felt unable to arrive at a diagnosis was 7; five lesions concerned a punch biopsy, two lesions had been mechanically damaged.
In 23 cases in which the panel had not reached an unequivocal diagnosis; the following differential diagnoses were given: Spitz naevus versus invasive melanoma (7), (dysplastic)naevus versus invasive melanoma (6), other special type of naevus versus invasive melanoma (5), melanoma in situ versus invasive melanoma (1). In four cases, other differential diagnoses were reported.
Of the 90 lesions without a referring pathologist's diagnosis the panel made an unequivocal diagnosis in 81 cases (including 21 invasive melanomas). Of the 131 lesions with a differential diagnosis by the referring pathologist, the panel had an unequivocal diagnosis in 122 cases (including 51 invasive melanomas). Of the 151 lesions with the referring diagnosis "suspect melanoma" the panel made an unequivocal diagnosis in 129 cases (including 71 invasive melanomas).
Overdiagnosis and underdiagnosis were defined and calculated from a perspective of clinical relevance including therapeutic consequences (see Materials and Methods).

***Overdiagnosis***

Of the 151 lesions classified by the referring pathologist as suspect melanoma, 55 lesions were overdiagnosed (Table III). Here it was taken into account that lesions classified in a pathology report as "suspect melanoma" will probably be regarded and treated by clinicians as if they were invasive melanoma. The panel classified 11 cases as common acquired naevus, 25 as Spitz naevus, 8 as other special type of naevus, 8 as dysplastic naevus and 3 as melanoma in situ. Of the 158 lesions classified by the referring pathologist as invasive melanoma, 22 lesions were overdiagnosed. The panel classified 3 cases as common acquired naevus, 10 as Spitz naevus, 4 as other special type of naevus, 2 as dysplastic naevus and 3 as melanoma in situ.

*Underdiagnosis*

Of the 84 lesions classified by the referring pathologist as common acquired naevus, 12 were classified by the panel as "suspect melanoma" or invasive melanoma (Table III). The numbers of the other underdiagnosed lesions were: Spitz naevus 25 of 157, other special type of naevus 7 of 54, dysplastic naevus 44 of 176 and melanoma in situ 20 of 46.

Table IV presents a summary of the diagnostic categories of referring pathologists versus panel pathologist(s) with the further grouping of diagnoses based on clinical relevance.

**Table IV: Summary of the diagnostic category of the referring pathologist versus the diagnostic category of the panel**

| Diagnosis of | Pathology Panel | | | | | Total |
|---|---|---|---|---|---|---|
| Referring pathologist | No diagn. | Benign/ DN/MIS | Diff. diagn. | Suspect mel. | Invasive mel. | |
| No diagn. | **1** | 56 | 2 | 6 | 21 | 86 |
| Benign/DN/MIS[a] | 3 | **391** | 11 | 23 | 85 | 513 |
| Diff. diagn. | 1 | 69 | **5** | 3 | 51 | 129 |
| Suspect mel. | 2 | 55 | 4 | **16** | 71 | 148 |
| Invasive mel. | 0 | 22 | 1 | 2 | **133** | 158 |
| Total | 7 | 593 | 23 | 50 | 361 | 1034[b] |

[a] lesions included: common acquired naevus, Spitz naevus, other special type of naevus, dysplastic naevus (DN) and melanoma in situ (MIS)

[b] lesions classified as "other" were excluded in this table

## DISCUSSION

Although the large majority of melanocytic tumours of the skin do not present significant diagnostic problems, a small minority of lesions are very difficult to diagnose (4,5,6). An expert pathology diagnosis of such cases may be of great clinical importance. In the present study, in 8% (90) of the lesions submitted, the referring pathologists could not provide a confident diagnosis, in 14% (151) there was suspicion of melanoma while in a further 12% (131) only a differential diagnosis, had been formulated. In these 372 lesions the panel was able to make an unequivocal diagnosis in 332 cases. The panel classified 7% (25) as suspect

melanoma and 38% (143) as invasive melanoma.

It is unclear how many cases of overdiagnosed invasive melanoma really occur in practice. In the present study overdiagnosis as "suspect melanoma" was found in 55 of 151 cases and as "invasive melanoma" in 22 of 158 cases. In the case of a diagnosis "suspect melanoma", a second opinion is always likely to be requested, as no certainty is given. In contrast, an incorrect but unequivocal diagnosis of invasive melanoma will not usually be submitted for pathology review, generally leading to overtreatment of the patient. Still, although an unequivocal diagnosis of invasive melanoma was reported by the referring pathologist in 22 cases, there must have been some element of doubt with regard to the diagnosis: otherwise, the case would not have been submitted for consultation.

Although the number of discordant diagnoses is quite high, the majority of diagnoses of the referring pathologists matched those of the panel. This might indicate a proper awareness of difficult cases in practice. Still, the only approach to come up with hard data is a systematic comparison of all diagnoses. In a documentation study of melanomas in one regional Dutch Comprehensive Cancer Centre East (Integraal Kankercentrum Oost, IKO), all invasive melanomas and melanomas in situ are reviewed by a member of the panel (DJR). Of the first 152 lesions, only one case diagnosed as lentigo maligna was classified by the panel member as common acquired naevus and three cases reported as melanoma in situ were diagnosed by the panel member as superficial spreading melanoma. These numbers are quite reassuring regarding the quality of the diagnosis. At the national level the use of the Dutch PALGA system makes it possible to review at a later stage cases of "thick" invasive melanomas which had not metastasized (7). Since most of the metastatic lesions will be verified histologically, a proportion of those thick invasive melanomas without documented metastases may be overdiagnosed naevi. These approaches may become a part of an integrated quality control programme in the future.

Underdiagnosis of invasive melanoma as benign lesion was noted in this study in 13-25% of the cases: 12 of 84 common acquired naevus, 25 of 157 Spitz naevus, 7 of 54 other special type of naevus, 44 of 176 dysplastic naevus and 20 of 46 melanoma in situ. Again, although these figures on discordant cases are quite high, the majority of the cases were concordant. This probably reflects a low threshold to submit cases to the panel and a proper identification of the group of difficult lesions.

A major problem in estimating the true number of underdiagnosed melanocytic lesions in general practice is the fact that most thin misdiagnosed invasive

melanomas will not metastasize and remain undetected as misclassified lesions. In a recent search for underdiagnosed cases covering about ten years in The Netherlands using the PALGA system, only a few cases of truly proven invasive melanomas "misdiagnosed" as Spitz naevus were uncovered (8). However, it remains unclear how many cases may have been given another key-diagnosis in the computerized system, after a case being recognized as a "mis-diagnosis", e.g., after metastasis.

Many lesions were diagnosed by the panel as Spitz naevus or as another special type of naevus. Also, of the 23 lesions with a differential diagnosis given by the panel, 7 lesions concerned the diagnosis Spitz naevus versus invasive melanoma, 6 lesions the diagnosis (dysplastic) naevus versus invasive melanoma and 5 lesions the diagnosis other special type of naevus versus invasive melanoma. Obviously, both referring pathologists and panel pathologists have difficulties in diagnosing such lesions (4,5,6,9,10). In terms of improving the general level of accuracy in melanoma diagnosis, it would appear to be most effective to concentrate postgraduate education on the specific problems concerning dysplastic naevus and Spitz naevus. A teaching slide set composed of difficult cases could be circulated among laboratories and discussed with an expert. During such discussions the technical quality of histological slides could also be addressed. At a later stage, test slide sets could be circulated in order to evaluate the diagnostic skill of the participating pathologists. Also quality monitoring may focus on the problems mentioned. Local laboratories can, together with consultation with more experienced colleagues in difficult cases, select representative lesions (i.e., not classical, but without a reason for consultation) for a second opinion (intra- or interinstitutional).

The procedure of consultation by individual panel members only is a result of the relatively large number of cases submitted and the need for a rapid response. A prompt response is greatly appreciated by the referring pathologist and the clinician. For this reason an independent opinion by all panel members on all cases is not feasible. As a compromise, only selected cases, which are considered difficult by the reviewing panel member, are sent to the other members for further evaluation. In addition, the regular panel meetings prove very useful in order to fine-tune the histopathological criteria and their interpretation. In almost every case a consensus could be reached, although this does not always mean an unequivocal diagnosis. In fact the panel, for mainly technical reasons, was not able to make a diagnosis in 7 cases and could make a differential diagnosis only in 23 cases and a diagnosis "suspect melanoma" in 50 cases. Here, additional techniques may provide relevant diagnostic information.

In this article the diagnosis of the panel is assumed to be the correct diagnosis. We realise that the panel too is subject to misinterpretation or observer bias. However, based on the specific experience acquired in this particular field of pathology the panel is more likely to change wrong diagnoses into correct ones than the other way around. Cases diagnosed as melanoma in situ or invasive melanoma are monitored through the cancer registry which will enable a clinical validation of the panel diagnosis. In addition, cases diagnosed as benign with a later presentation of melanoma metastasis, can be traced systematically through the Dutch pathology data bank PALGA.

So far, there has been no opportunity to perform an interobserver study among all panel members on submitted, or otherwise selected, difficult cases. However, in a recent extended interobserver study on melanocytic lesions among 10 (dermato)pathologists in the melanocytic field in Europe, the best overall inter-observer agreement was between two members of the panel who participated in that study (11). Furthermore, in an earlier independent review of a set of 104 melanocytic lesions (not especially difficult cases), the pair-wise interobserver agreement among the three panelmembers was good (agreement $> 95\%$ and kappa $> 0.80$; unpublished results, PEJdW) compared to other studies. Even so, an interobserver study on the lesions, received for consultation as described here, needs to be performed as this is a specific subgroup of cases. The lesions are difficult to diagnose, more often located in the head and neck region and the patients tend to be younger. Such a study may yield more specific information on the problems encountered in this area of pathology.

In this paper the diagnoses of referring pathologists and the Pathology Panel of the Dutch Melanoma Working Party were compared. The rates of overdiagnosis and underdiagnosis (together with incorrect treatment) are shown to be quite high. There is no reason to believe that such rates of diagnostic problems exist only in The Netherlands. Difficulties in diagnosing melanocytic lesions are well known in the international literature (4,5,6,9,10) but they are described in a more qualitative manner. In the three years analysed, the panel received some requests from pathologists abroad, amongst which misdiagnoses were also found.

In summary, our data indicate that a second opinion given by pathologists, experienced in the diagnosis of cutaneous melanocytic lesions, is useful for the quality assurance of the pathology diagnosis. Uncertainty in the assessment of diagnoses and over- or underdiagnosis are serious problems that deserve to receive continuous attention. The quality of melanoma pathology can be improved by expert

review and by continuing education targeted at the specific problems signalled.

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# CHAPTER 3

## Validity of the histopathological criteria used for diagnosing dysplastic naevi

### An interobserver study by the pathology subgroup of the EORTC Malignant Melanoma Cooperative Group

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**ABSTRACT**

Ten (dermato)pathologists studied fifty cutaneous melanocytic lesions including common naevocellular naevi, dysplastic naevi (D.N.), melanomas in situ and invasive primary melanomas, with emphasis on the histological criteria of D.N. Using a standardized form, 20 defined histopathological features were scored (semi)quantitatively. Concordance of diagnosis, efficacy and reproducibility of features were investigated. D.N. were distinguished well from the other entities (mean $P_o$ 0.87). Agreement on the degree of atypia of D.N. was low. The reproducibility of the scoring was best for the following features: "irregular nests, lymphohistiocytic infiltrate, marked junctional proliferation and large nuclei". The overall values of these features to discriminate between D.N. and non-dysplastic naevi were better than for the other features studied.

Using the presence of at least 3 of the 4 features as a condition for the diagnosis of D.N., values for sensitivity, specificity, positive and negative predictive values were 0.86, 0.91, 0.96 and 0.73, respectively. On the basis of the results these features seem best suited as histological criteria for the diagnosis of D.N. *(Eur J Cancer 1993; 29a: 831-839).*

## INTRODUCTION

The dysplastic naevus (D.N.) is considered a precursor to cutaneous melanoma. In addition, it may serve as a marker of an increased risk of developing melanoma. Clinical hallmarks often encountered are a largest diameter of 5 mm or more, asymmetry, irregular borders and a variegated colour (1). Histopathological examination of selected lesions is needed to confirm a clinical diagnosis of D.N. and to detect or exclude early melanoma. Elder et al have first described the histopathological criteria for the diagnosis of D.N. (2,3). They consist of features of cytological atypia, architectural atypia and stromal changes. Later, these features were debated and others were added (4-19). At present, it is not settled which of the histological criteria are obligatory for the diagnosis, and if so, to what degree and extent a feature should be present. Issues related to this problem are differences in the appreciation of the concept of D.N. as an entity and differences in the final interpretation of the observations. Basically however, the selection and weight of a feature for a diagnostic purpose rests on its validity in terms of the discriminatory value (efficacy) and the reproducible recognition among observers. With regard to D.N., research into the latter matter is sparse and concerns only some histological features (16). As it is important to provide clinicians with a consistent histopathological diagnosis the pathology subgroup of the EORTC Malignant Melanoma Cooperative Group, under the auspices of the E.C. Concerted Action on Melanoma, commenced the present study. The validity of the morphological features which have been propagated was investigated in relation to their interpretation in terms of concordance of diagnosis among observers. The results obtained are compared with other studies with respect to reproducibility (16) and efficacy (13) of features and concordance of diagnosis (19). Although this work may be considered a pilot study, provisional guidelines are given on the histopathological classification of D.N., which also may direct future research among (dermato)pathologists in a routine setting.

## MATERIALS AND METHODS

### *Histological slides*

A set of 50 histological slides was used, taken from the files of the Pathology Panel of the Dutch Melanoma Working Party. Based on their individual diagnoses on all 50 slides, the set consisted of nine non-dysplastic benign melanocytic lesions, 25 dysplastic naevi, seven melanomas in situ and nine invasive melanomas. Thus, a wide spectrum of melanocytic lesions was included although

enriched for certain categories. As the distinction between benign versus malignant can serve as a quality check of the observers, rather a lot of premalignant and malignant lesions were included. D.N. were the lesions of principal interest in this study. According to the Dutch Panel, following the criteria of Steijlen et al (13), the set of D.N. consisted of approximately equal numbers of lesions with slight, moderate and marked atypia.

*Observers*

Ten (dermato-)pathologists participated as an observer. The same slides were sent to each observer and evaluated independently within a period of three weeks.

*Morphological features and histopathological evaluation*

A standardized form for histopathological evaluation was used, listing morphological features based on the literature and personal experience. In addition, criteria for melanoma in situ and invasive melanoma were listed (Appendix I). A lesion could be diagnosed as common naevocellular naevus, common naevocellular naevus with minor abnormal features, D.N., melanoma in situ, superficial spreading melanoma, other lesion. In order to facilitate a consistent appreciation of the morphological features a list with detailed definitions of the criteria used was added (Appendix II). No set of criteria for the diagnosis D.N. or other diagnoses was proposed. The form was discussed and accepted by the observers before circulation of the histological slides. One slide per lesion was studied. In most cases several sections were mounted on the slide. No clinical data were provided.

*Statistical methods*

The interobserver agreement was studied by calculating the proportion of agreement ($P_o$) and the Kappa-coefficient (the proportion of agreement in excess of what is expected by chance, see Appendix III). The measures of agreement in diagnosis were also calculated when certain categories were combined. Both the mutual agreement in diagnosis of all observers and the agreement with the panel diagnosis were determined. The EORTC-panel diagnosis was considered to be the diagnosis of a majority of the 10 observers. The reproducibility of the features was studied by calculating Pearson correlation coefficients and by applying Kappa statistics. The discriminating value of the features was described using discriminant analyses and Fisher exact tests. Sensitivity, specificity, positive and negative predictive value for the features with respect to the panel diagnoses were calculated.

## RESULTS

Results reported here mainly concern the issues related to the diagnosis of D.N.

### *Concordance of diagnosis*

Before further analysis of the data, the observers' diagnoses were a posteriori classified in one of the six categories mentioned in Table I.

**Table I: A posteriori classification of the observers' diagnoses**

Category 1
- Benign proliferations of melanocytes, except "Spitz naevus" and "freckle".
  It includes: common naevocellular naevus
  congenital naevus
  lentigo
  blue naevus
  combined naevus

Category 2
- Common naevocellular naevus with minor abnormal features.

Category 3
- Dysplastic naevus.

Category 4
- Melanoma in situ.
  It includes: in situ SSM*
  in situ ALM

Category 5
- Invasive melanoma.
  It includes: SSM
  NM
  LMM
  ALM
  unclassified melanoma
  acral SSM
  cutaneous melanoma metastasis

Category 6
- "Other diagnoses", including Spitz naevus (8x), freckle (7x), hyperpigmentation of basal keratinocytes (1x), functional hyperpigmentation (2x), pigmented Bowen's disease (1x), mastocytosis (1x), no diagnosis (4x).

* SSM: superficial spreading melanoma; NM: modular melanoma; LMM: lentigo maligna melanoma; ALM: acrolentiginous melanoma

All observers agreed on 10 slides, while at least eight observers agreed on 23 slides, and at least five observers agreed on 46 slides. The distribution of the diagnoses per observer is given in Table II. It illustrates the interobserver variation in the diagnoses made. All observers scored one or more lesions in category 2 (mean 6.5), but only in one case did a majority agree on this diagnosis.

**Table II: Distribution of observers' diagnoses**

| Observer | Number of slides per diagnostic category | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| a | 13 | 8 | 13 | 2 | 10 | 4 |
| b | 8 | 10 | 17 | 4 | 9 | 2 |
| c | 7 | 4 | 21 | 4 | 12 | 2 |
| d | 7 | 1 | 25 | 5 | 11 | 1 |
| e | 11 | 9 | 14 | 2 | 12 | 2 |
| f | 5 | 2 | 27 | 4 | 10 | 2 |
| g | 8 | 11 | 12 | 6 | 11 | 2 |
| h | 19 | 5 | 4 | 10 | 10 | 2 |
| i | 15 | 6 | 12 | 7 | 7 | 3 |
| j | 9 | 9 | 15 | 6 | 7 | 4 |
| a-j mean | 10.2 | 6.5 | 16 | 5 | 9.9 | 2.4 |
| s.d. | 4.3 | 3.4 | 6.8 | 2.4 | 1.8 | 0.8 |
| EORTC panel* | 9 | 1 | 23 | 6 | 9 | 2 |

* The diagnosis of a majority of the 10 observers

As is shown in Table IIIA the mean $P_o$ and Kappa values for the comparison of benign vs. malignant lesions are high, i.e. 0.90 and 0.76, respectively. Also, mean values for the comparison II are relatively high, i.e. 0.79 and 0.58. These figures represent the means of 45 interobserver comparences. When the diagnoses of one observer were compared to the diagnoses of a majority of the 10 observers ("panel diagnoses"), the values were higher (Table IIIB). Stratification of the diagnostic categories coincides with a decrease of the $P_o$ and Kappa values,

but for all diagnoses taken separately (analysis V) mean $P_o$ and Kappa values are acceptable: 0.70 and 0.61, respectively. A low level of agreement ($P_o$ mean = 0.28; s.d. = 0.16; 45 observer pairs) was found on the degree of histopathological atypia between pairs of observers. This comparison was made if both observers of a pair classified the lesion as either common naevocellular naevus with minor abnormal features or as a D.N., thus constituting 4 levels of atypia, that could be scored (appendix I, items 38 and 39).

***Discriminative value***

The sensitivity, specificity, positive and negative predictive value of the features discriminating between a non-dysplastic naevus and a D.N. are given in Table IV. Highest values for sensitivity were found for the following features: percentage of melanocytic cells with at least one feature of cytological atypia, irregular nests, lymphohistiocytic infiltrate, large nuclei, elongated rete ridges and nuclear pleomorphism. Highest values for specificity were found for the following features: abnormal distribution of melanin, marked junctional proliferation, irregular nests, neovascularisation, lamellar fibroplasia and shoulder phenomenon. Highest values for positive predictive value were found for: irregular nests, marked junctional proliferation, lamellar fibroplasia, nuclear polymorphism and shoulder phenomenon. Highest values for negative predictive value were found for: irregular nests, % melanocytic cells with at least one of the cytological atypia features, large nuclei, nuclear polymorphism, and lymphohistiocytic infiltrate. For these analyses panel diagnoses were used. Scoring of the features was dichotomized. Dichotomization, other than into present vs. absent, resulted in lower figures. From two observers some data were missing, which prohibited a meaningful analysis.

Discriminant analysis showed that using more than three to four features had no further discriminating value. Although the most discriminating features varied from observer to observer, certain features were more often among the top four. Table V shows the overall hierarchy of the best discriminating features. The average $R^2$-values for these features varied from 0.65 (for irregular nests) to 0.30 (for large cytoplasma). The hierarchical order obtained by discriminant analysis correlated well with the order obtained when applying a Fisher exact test, ranking the features according to significance (best P-values). Features not listed, but with a relatively high discriminating value in a single observer were: bridges of melanocytes, lymphohistiocytic infiltrate, lamellar fibroplasia, dust-like melanin and elongated rete ridges.

**Table III: Reproducibility of diagnosis expressed in $P_o$ and Kappa values***

**A.** Means and s.d. of 45 $P_o$ values and Kappa values of 45 comparisons of two observers (ab .....ij)

| | $P_o$ | | | | | Kappa | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | I | II | III | IV | V | I | II | III | IV | V |
| mean | 0.90 | .79 | .69 | .63 | .57 | 0.76 | .58 | .57 | .50 | .45 |
| s.d. | .06 | .09 | .10 | .09 | .09 | .13 | .17 | .12 | .10 | .10 |

**B.** $P_o$ and Kappa of comparisons between observer and "panel" diagnosis for each of observers a-j

| | $P_o$ | | | | | Kappa | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | I | II | III | IV | V | I | II | III | IV | V |
| a-panel | 0.94 | .90 | .82 | .70 | .70 | 0.85 | .80 | .75 | .60 | .62 |
| b-panel | .96 | .86 | .86 | .76 | .70 | .90 | .72 | .79 | .67 | .61 |
| c-panel | .82 | .82 | .72 | .76 | .70 | .58 | .64 | .59 | .66 | .59 |
| d-panel | .98 | .92 | .82 | .82 | .80 | .95 | .84 | .73 | .74 | .71 |
| e-panel | .94 | .86 | .78 | .66 | .62 | .85 | .72 | .68 | .54 | .54 |
| f-panel | .94 | .90 | .82 | .84 | .80 | .85 | .80 | .73 | .76 | .71 |
| g-panel | .96 | .94 | .88 | .72 | .70 | .91 | .87 | .83 | .63 | .62 |
| h-panel | .92 | .70 | .62 | .54 | .54 | .82 | .38 | .52 | .43 | .45 |
| i-panel | .92 | .82 | .74 | .66 | .66 | .80 | .68 | .64 | .55 | .57 |
| j-panel | .96 | .98 | .92 | .78 | .78 | .90 | .96 | .88 | .70 | .71 |
| mean | .93 | .87 | .80 | .72 | .70 | .84 | .74 | .71 | .63 | .61 |
| s.d. | .04 | .08 | .09 | .09 | .08 | .10 | .16 | .11 | .10 | .08 |

* Comparisons based on clustering of diagnoses (categories of Table I):

| | | |
|---|---|---|
| I | : [1,2,3,6] vs [4,5] | benign/malignant |
| II | : [2,3] vs [1,4,5,6] | |
| III | : [1] vs [2,3] vs [4] vs [5] vs [6] | categories 2 and 3 together |
| IV | : [1,2] vs [3] vs [4] vs [5] vs [6] | categories 1 and 2 together |
| V | : [1] vs [2] vs [3] vs [4] vs [5] vs [6] | all diagnoses separately |

**Table IV: Sensitivity, specificity, positive and negative predictive value of histopathological features in "dysplastic" vs. "non-dysplastic" naevi***

| Feature | sensitivity | specificity | positive predictive value | negative predictive value |
|---|---|---|---|---|
| - large nuclei | 0.87 | 0.67 | 0.87 | 0.68 |
| - nuclear hyperchromasia | .72 | .71 | .86 | .51 |
| - nuclear polymorphism | .87 | .67 | .87 | .68 |
| - prominent nucleoli | .67 | .80 | .89 | .50 |
| - large cytoplasm | .81 | .74 | .88 | .62 |
| - % melanocytic cells with at least one of the cytological atypia features | .94 | .45 | .81 | .76 |
| - irregular nests | .89 | .84 | .93 | .76 |
| - marked junctional proliferation | .71 | .84 | .91 | .55 |
| - dust-like melanin | .62 | .75 | .86 | .45 |
| - elongated rete ridges | .87 | .17 | .71 | .35 |
| - loss of preference for rete ridge tips | .67 | .77 | .80 | .63 |
| - "shoulder phenomenon" | .70 | .80 | .89 | .53 |
| - abnormal distribution of pigment | .33 | .88 | .87 | .36 |
| - lymphohistiocytic infiltrate | .88 | .52 | .81 | .65 |
| - lamellar fibroplasia | .70 | .81 | .90 | .53 |
| - neovascularisation | .51 | .83 | .88 | .42 |

* panel diagnoses were used (category 1 and 2 vs. 3); for each feature the total of the data from eight observers was used to calculate sensitivity, specificity, positive and negative predictive value; scoring of features was dichotomized (present vs. absent); data from two observers were incomplete.

**Table V: Overall hierarchy in discriminating value of histopathological features for the discrimination between "normal" naevi and "dysplastic" naevi***

| |
|---|
| - irregular nests |
| - marked junctional proliferation |
| - large nuclei |
| - shoulder phenomenon |
| - nuclear hyperchromasia |
| - loss of preference for rete ridge tips |
| - nuclear polymorphism |
| - large cytoplasm |

* Hierarchy based on discriminant analyses and Fisher exact tests. Some features not listed had a relatively high value in a single observer e.g. bridges of melanocytes, lymphohistiocytic infiltrate, lamellar fibroplasia, dust-like melanin, elongated rete ridges

***Reproducibility of feature scoring***

The concordance in the non-dichotomized scoring of the features among eight observers can be found in Table VI. From two observers some data were missing, which prohibited a meaningful analysis. The results of the analysis of all diagnoses (category 1 to 6) did not differ from those of the analysis restricted to category 1, 2 and 3 only. Highest mean Pearson correlation coefficients ($\geq 0.65$) with also the highest minimum values for a single observer (see range), were found for the following features: lymphohistiocytic infiltrate, irregular nests, and marked junctional proliferation. Counting of the number of "bridges of melanocytes" was hardly reproducible (not shown).

Based on the foregoing results features were selected and Kappa values calculated for the agreement on their presence or absence in benign lesions (i.e. categories 1, 2 and 3). Best values were obtained for: irregular nests, lymphohistiocytic infiltrate, marked junctional proliferation, and large nuclei. The results are shown in Table VII. The highest Kappa value was found for the first feature mentioned. Values were not higher when another dichotomization then present vs. absent was used. The histopathological features are illustrated in Figs 1 and 2. Figs 3 and 4 show photomicrographs of lesions which were classified as common naevocellular naevus and melanoma in situ, respectively.

**Table VI: Comparison of the scoring of histopathological features among eight observers using Pearson correlation coefficients[a]**

| Feature | Correlation coefficient | | | |
|---|---|---|---|---|
| | A[b] | | B | |
| | mean | range | mean | range |
| large nuclei | 0.53 | 0.42-.68 | 0.53 | 0.42-.69 |
| nuclear hyperchromasia | .45 | .10-.59 | .46 | .10-.59 |
| nuclear polymorphism | .57 | .34-.71 | .58 | .34-.71 |
| prominent nucleoli | .43 | .27-.67 | .43 | .27-.67 |
| large cytoplasm | .53 | .26-.79 | .53 | .26-.79 |
| % melanocytic cells with at least one cytological atypia feature | .52 | .19-.80 | .52 | .19-.80 |
| irregular nests | .72 | .56-.81 | .72 | .56-.81 |
| marked junctional proliferation | .65 | .50-.80 | .65 | .50-.88 |
| dust-like melanin | .41 | .00-.77 | .41 | .00-.77 |
| elongated rete ridges | .53 | .31-.78 | .53 | .31-.78 |
| loss of preference for rete ridge tips | .48 | .06-.74 | .48 | .06-.74 |
| "shoulder phenomenon" | .60 | .22-.74 | .60 | .22-.74 |
| abnormal distribution of pigment | .46 | .18-.73 | .46 | .18-.73 |
| lymphohistiocytic infiltrate | .79 | .63-.89 | .79 | .63-.89 |
| lamellar fibroplasia | .56 | .43-.69 | .56 | .43-.69 |
| neovascularization | .28 | .00-.55 | .28 | .00-.55 |

[a] Per observer the score (not dichotomized) of that observer was compared to the mean of the score of seven other observers; data from two observers were incomplete; per feature the mean and range of the results from eight observers is presented.

[b] A: all categories of Table I included; B: limited to categories 1, 2 and 3.

**Table VII: Kappa values for the agreement on presence/absence of four features***

| Observer | feature | | | |
|---|---|---|---|---|
| | irregular nests | lymphohistio-cytic infiltrate | marked junctional proliferation | large nuclei |
| a | 0.60 | 0.38 | 0.36 | 0.40 |
| b | .65 | .49 | .40 | .44 |
| c | .52 | .40 | .46 | .28 |
| d | .60 | .56 | .28 | .46 |
| e | .38 | .50 | .47 | .37 |
| g | .67 | .45 | .55 | .48 |
| h | .54 | .56 | .31 | .19 |
| j | .68 | .38 | .47 | .33 |
| mean a-j | .58 | .47 | .41 | .37 |
| s.d. | .10 | .07 | .09 | .10 |

* - Per observer the mean of kappa's of that observer compared with each of seven others is presented.
- Provided the own diagnosis of the two compared observers was 1, 2 or 3.
- Dichotomized: present vs. absent

## DISCUSSION

A first condition for a reliable diagnostic test is that the result is reproducible on a given specimen. As the scoring of multiple histopathological features in diagnosing melanocytic lesions is such a test, we investigated the reproducibility of the scoring. No clinical data were provided. The four best reproducible features were: irregular nests, lymphohistiocytic infiltrate, marked junctional proliferation and large nuclei. Although the Kappa values of these features are not very high, they are still better than those of the other features examined. The fact that, when another dichotomization or further stratification was used, values became lower, indicates that reproducibility is best when a simple present versus absent dichotomization is used. Similarly, Ahmed et al (16) found low Kappa values, using four levels that could be scored for junctional activity, irregular nests, dusty melanin and large melanocytic nuclei.

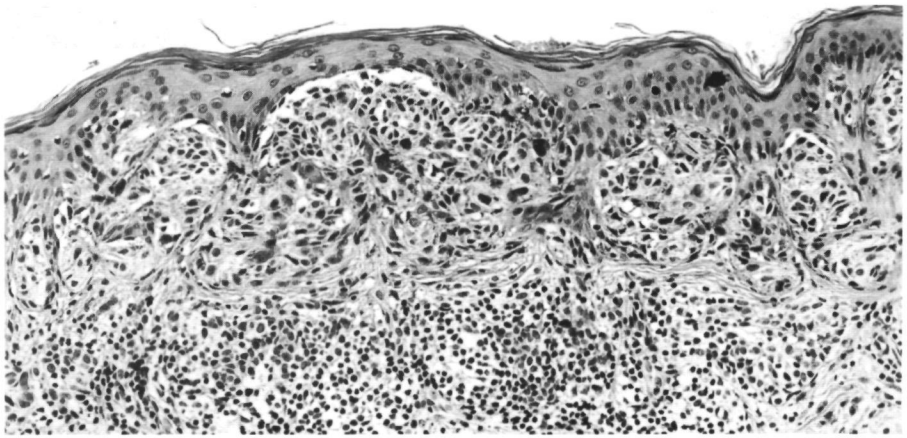

**Figure 1: Dysplastic naevus.**

Photomicrograph of a dysplastic naevus, showing marked junctional activity, irregular nests, and lymphocytic infiltrate. At this magnification, large nuclei cannot be appreciated properly.

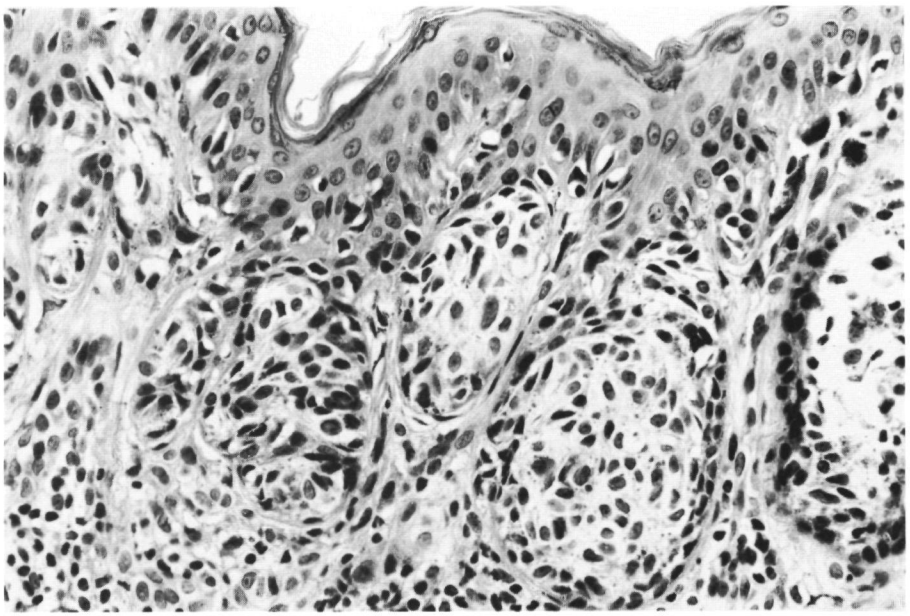

**Figure 2: Dysplastic naevus.**

Higher magnification of the lesion shown in Fig. 1. Note several melanocytic nuclei that are larger than those of adjacent keratinocytes.

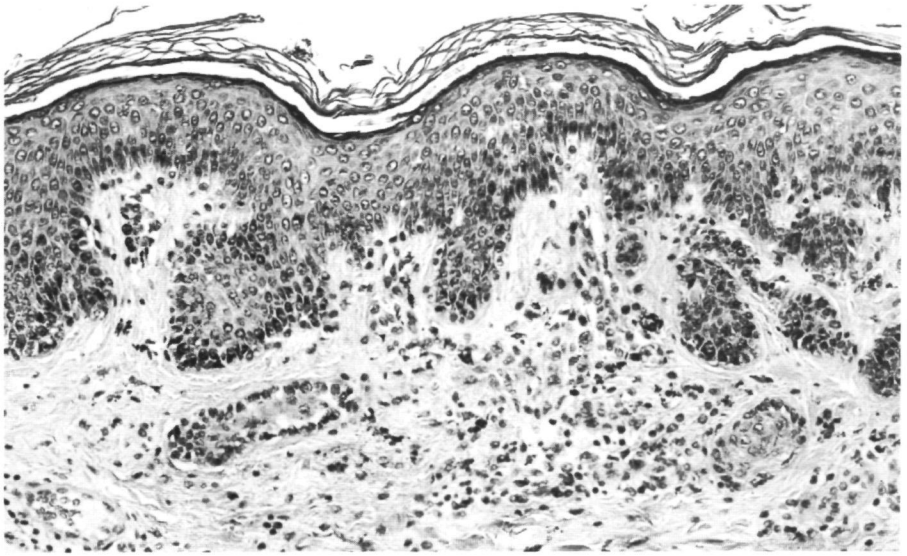

**Figure 3: Common naevocellular naevus.**

Photomicrograph of a common compound naevocellular naevus, showing slight architectural abnormalities.

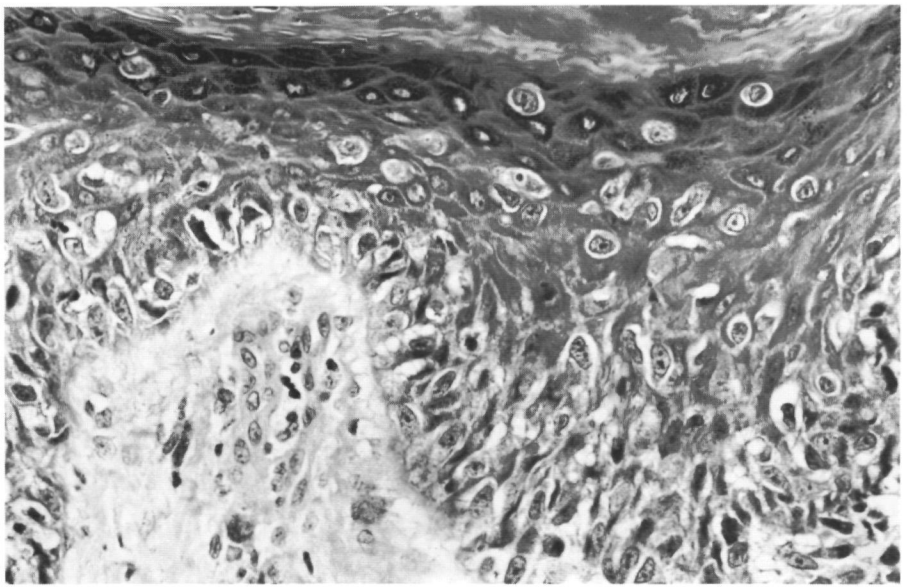

**Figure 4: Melanoma in situ.**

Photomicrograph of a melanoma in situ: pagetoid growth of atypical melanocytes.

Given these basic values on the reproducibility of the scoring, examination of the validity of each of the four features to discriminate between dysplastic naevi and common naevi, showed that three of them scored the highest in the discriminant analysis. Also, their values for sensitivy, specificity, positive and negative value were among the best. This is in agreement with the study by Steijlen et al (13), who found the best efficacy for irregular nests, marked junctional activity, large melanocytic nuclei and dust-like melanin.

In 21 of the 23 lesions that in the present study were diagnosed as D.N. (panel diagnoses), all four best reproducible features were present, as determined by the score of the majority of the observers. However, features were "missed" in a number of cases by individual observers, illustrating the suboptimal reproducibility of the feature scoring. Based on these data and the fact that the discriminant analysis showed no further value in the discrimination between "common" and "dysplastic" naevi when using more than four features, we examined the discriminative value of combinations of these features to distinguish D.N. from common normal naevi. Using the presence of all four features as a diagnostic prerequisite for D.N., values for sensitivity, specificity, positive and negative predictive value were 0.53, 0.93, 0.95 and 0.45 respectively. Thus, the missing of one feature by an observer would imply that D.N. would not be diagnosed as such.

However, using the presence of at least three of the four features as a diagnostic prerequisite for D.N., values for sensitivity, specificity, positive and negative predictive values were 0.86, 0.91, 0.96 and 0.73, respectively. This implies that a very acceptable efficacy can be reached with a limited set of reproducible criteria, which has advantages in terms of learnibility and applicability. For this practical reason we propose lesions be diagnosed as D.N. if at least three of the four features mentioned are present. In the lesions diagnosed as D.N. by the panel, nuclear atypia was nearly always scored as present by the individual observers. In our opinion lesions with one or two of the features should be classified as a common naevocellular naevus, while mentioning the minor abnormal features in the detailed microscopic report or note. They should not be diagnosed as a separate entity. For the 10 observers, the mean value for the correct classification with respect to the panel diagnosis in either category 1 and 2 or in category 3 was 88%, using the proposed approach summarised in Table VIII. The morphological features mentioned are shown in Figures 1 and 2. In this approach grading of atypia in D.N. is not recommended since it is not reproducible. The features relevant for the differential diagnosis of D.N.,

**Table VIII: Proposed diagnostic approach**

| Morphological features | Diagnosis |
|---|---|
| No or less than three of the features mentioned below for dysplastic naevus | Common naevocellular naevus |
| Three or more of the following features<br>- marked junctional proliferation<br>- irregular nests<br>- large nuclei<br>- lymphohistiocytic infiltrate | Dysplastic naevus |
| Pagetoid growth<br>Continuous junctional proliferation | Melanoma in situ |
| Pagetoid growth<br>Continuous junctional proliferation<br>Invasion of markedly atypical melanocytes into the dermis | Superficial speading melanoma |

melanoma in situ and superficial spreading melanoma are also included in Table VIII.

"Marked junctional activity" and "irregular nests" both reflect architectural atypia, "large nuclei" reflects cytological atypia and "lymphohistiocytic infiltrate" reflects stromal changes. Comparing our study with that published by Clemente et al (19), we feel that "marked junctional activity" is similar to their major criterion "basilar proliferation of atypical melanocytes (extending at least three rete ridges or "pegs" beyond any dermal naevocellular component)". In addition, our "irregular nests" is comparable to their other major criterion "organisation of the melanocytic proliferation in a lentiginous or epitheloid-cell pattern". Our feature "large melanocytic nuclei" is included in their first mentioned criterion, as the term "atypical melanocytes" is used. Of their minor criteria, i.e. "lamellar fibrosis or concentric eosinophilic fibrosis", "neovascularisation", "inflammatory response", and "fusion of rete ridges", the feature "lymphohistiocytic infiltrate" was most reproducible in our study. Clemente et al (19) found good concordance of diagnosis, using a previously agreed upon set of criteria. In the present study, no set of criteria was suggested by and to the observers. Emphasis was put on the defenition of the features, which were scored. The interpretation was left to the

observers. Doing so, we also found a very acceptable concordance of the diagnosis for most observers.

In the present study the matter of the preferred nomenclature of D.N. was not addressed. The most important issue to our opinion is that dermatologists, pathologists and dermatopathologists formulate a reproducible scheme for diagnosing and reporting these naevi. We feel that studies on the efficacy and reproducibility of histopathological features for diagnosing D.N. are more instrumental to solve this issue than semantic discussions on the nomenclature of the lesion.

In conclusion, our study indicates that it is possible to reliably diagnose D.N. by histopathological examination. A reproducible set of criteria with, in theory, good discriminating value can be compiled. Our study was set up as a first step to standardize the histopathological diagnosis of D.N. among European centres. Further studies should reveal whether our proposed diagnostic approach can add to a consistent diagnosis of D.N., among pathologists and dermatopathologist in centers, who may coordinate continuing education and quality control, and, more important, among those in a routine setting.

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## APPENDIX I

## EORTC-MMCG PATHOLOGY FORM ON THE DIAGNOSIS OF DYSPLASTIC NAEVUS

| | | | |
|---|---|---|---|
| SLIDE NUMBER | : | ☐☐ | 1-2 |
| Date (day, month, year) | : | ☐☐☐☐☐☐ | 3-8 |
| Reader (write or stamp) | : | ☐☐ | 9-10 |

### HISTOPATHOLOGICAL EVALUATION

General remarks:

1) The criteria for cellular and architectural atypia ad A concern the intra-epidermal (including the junctional) component only.
2) If asked for, the proportion of atypical structures (e.g. melanocytic cells, nests) as part of the total in the lesion, is given in a percentage estimated in one of the following classes:
   0% (code: 0), 1-10% (code: 1), 11-50% (code 2), 51-100% (code 3).
3) Further definition of the criteria: see addendum.

### A) MAJOR CRITERIA

1) Cytological atypia

| | | |
|---|---|---|
| large nuclei (percentage code) | ☐ | 11 |
| nuclear hyperchromasia (percentage code) | ☐ | 12 |
| nuclear polymorphism (percentage code) | ☐ | 13 |
| prominent nucleoli (percentage code) | ☐ | 14 |
| large cytoplasma | ☐ | 15 |
| What is the estimated percentage of melanocytic cells with at least one of the cytological atypia features? (percentage code) | ☐ | 16 |

2) Architectural atypia

| | | |
|---|---|---|
| irregular nests (percentage code) | ☐ | 17 |
| marked junctional proliferation (yes=1, no=0) | ☐ | 18 |

### B) MINOR CRITERIA

3) Cytological change

| | | |
|---|---|---|
| dust-like melanin (percentage code) | ☐ | 19 |

4) Architectural changes

| | | |
|---|---|---|
| elongated rete ridges (yes=1, no=0) | ☐ | 20 |
| bridges of melanocytes (maximal no. per section) | ☐☐ | 21-22 |
| loss of preference for rete ridge tips (yes=1, no=0) | ☐ | 23 |
| "shoulder" phenomenon (yes=1, no=0) | ☐ | 24 |
| abnormal distribution of pigment (yes=1, no=0) | ☐ | 25 |
| lymphohistiocytic infiltrate (see addendum) | ☐ | 26 |
| lamellar fibroplasia (yes=1, no=0) | ☐ | 27 |
| neovascularisation (yes=1, no=0) | ☐ | 28 |

SLIDE NUMBER : ☐☐ 29-30

Reader (write or stamp) : ☐☐ 31-32

## C. CRITERIA FOR MELANOMA (IN SITU)

- continuous atypical melanocytic proliferation (yes=1, no=0) ☐ 33
- pagetoid cells present (yes=1, no=0) ☐ 34
- ascension of atypical melanocytes reaching granular layer (mention maximal number found in one section) ☐☐ 35-36
- lack of maturation (yes=1, no=0) ☐ 37
  (no decrease in nuclear size of dermal melanocytic cells as compared to junctional component)

**DIAGNOSIS** (according to your own criteria) ☐ 38

| | |
|---|---|
| Common naevocellular naevus | = 1 |
| Common naevocellular naevus with minor abnormal features | = 2 |
| Dysplastic naevus | = 3 |
| Melanoma in situ | = 4 |
| SSM | = 5 |
| Other, please specify ..... | = 6 |

If a dysplastic naevus please indicate degree of atypia: (according to your own interpretation) ☐ 39

| | |
|---|---|
| not applicable | = 0 |
| slight | = 1 |
| moderate | = 2 |
| marked | = 3 |

**CONTEXT OF LESION** ☐ 40

| | |
|---|---|
| Not applicable | = 0 |
| Dysplastic naevus ass. with common naevocellular naevus | = 1 |
| Melanoma in situ ass. with common naevocellular naevus | = 2 |
| Melanoma in situ ass. with dysplastic naevus | = 3 |
| SSM associated with common naevocellular naevus | = 4 |
| SSM associated with dysplastic naevus | = 5 |

If the lesion would not have been completely resected, would you consider re-excision? (yes=1, no=0) ☐ 41

**COMMENTS:**

## APPENDIX II
## ADDENDUM TO THE EORTC-MMCG PATHOLOGY FORM ON THE DIAGNOSIS OF DYSPLASTIC NAEVUS

Further definition of the criteria

| | | |
|---|---|---|
| Large nuclei | : | nuclear size ≥ nuclei of basal keratinocytes |
| Nuclear hyperchromasia | : | nuclear density ≥ nuclei of lymphocytes |
| Nuclear polymorphism | : | irregular size/shape/orientation |
| Prominent nucleoli | : | nucleolar size ≥ one-third of erythrocytes |
| Large cytoplasma | : | as compared to melanocytes in perilesional skin |
| Irregular nests | : | irregular size/shape |
| Marked junctional proliferation | : | number of melanocytes (either as single cells or loose aggregates) ≥ number of basal keratinocytes, along at least three adjacent rete ridges (nestlike or lentiginous) |
| Dust-like melanine | : | finely and equally dispersed grey-brown pigment |
| Elongated rete ridges | : | as compared to those in perilesional epidermis |
| Bridges of melanocytes | : | rete rigdes connected by bridging nests of melanocytes |
| Loss of preference for rete ridge tips | : | arrangement of solitary units and/or nests of melanocytes at the lateral side of the rete ridges and/or over the top of dermal papillae |
| Shoulder phenomenon | : | extension of junctional component over at least three rete ridges beyond the dermal naevocellular component |
| Abnormal pigment distribution | : | retention of melanin throughout dermal naevocellular component |
| Lymphohistiocytic infiltrate | : | 0=no; 1=slight, perivascular; 2=moderate, aggregate-like; 3=marked, band-like |
| Lamellar fibroplasia | : | as compared to the papillary dermis in perilesional skin |
| Neovascularization | : | as compared to the papillary dermis in perilesional skin. |
| Pagetoid cells | : | abnormal melanocytes with abundant pale cytoplasma containing dusty melanin |

## APPENDIX III

## ADDENDUM TO THE EORTC-MMCG PATHOLOGY FORM ON THE DIAGNOSIS OF DYSPLATIC NAEVUS

The observer agreement for categorical data*

$P_O$: represents the observed proportion of agreement between two observers (the observed proportion of subjects with equal diagnosis for the two observers).

K: represents the proportional excess of agreement beyond what is to be expected under independence. The coefficient kappa is defined by

$$K = \frac{o - e}{1 - e}$$

o = observed proportion of agreement
e = expected proportion of agreement

* Cohen, J.
A coeficient of agreement for nominal scales.
*Educational and Psychological Measurement 1960; 20: 37-46.*

Quality of Kappa**

| | |
|---|---|
| ≤ 0.20 | poor/slight |
| 0.21 - 0.40 | fair |
| 0.41 - 0.60 | moderate |
| 0.61 - 0.80 | substantial |
| 0.81 - 0.99 | almost perfect |
| 1.00 | perfect |

** Landis J.R. and Koch G.G.
The measurement of observer agreement for categorial data.
*Biometrics 1977; 33: 159-174*

# CHAPTER 4

# Increasing epidermal growth factor receptor expression in human melanocytic tumor progression

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**Journal of Investigative Dermatology 1992; 99: 168-173**

**ABSTRACT**

Different results have been reported on the expression of epidermal growth factor receptor (EGFR) in human melanocytic lesions, which may be due to different methodological approaches. Therefore, we compared EGFR expression in six human melanoma cell lines by utilizing the monoclonal antibodies 2E9, 425 and 225, applying four immunocytochemical staining procedures. The results were compared with those obtained by a multiple point ligand binding assay. In addition, Northern blot analysis was performed.

A three-step immunoperoxidase method using the monoclonal antibody 2E9 proved the most sensitive. Staining intensities, estimated semiquantitatively, correlated well with the quantitative data obtained by the ligand binding assay. Expression on the mRNA level was also in agreement with these results.

Immunohistochemical staining of a large series of human cutaneous melanocytic lesions using the method selected showed differential EGFR expression in various stages of melanocytic tumour progression: 19% of common nevocellular nevi, 61% of dysplastic nevi, 89% of primary cutaneous melanomas and 91% of melanoma metastases showed staining of the melanocytic cells. Intralesional heterogeneity of EGFR expression was present. Although the mean percentage of positive melanocytic cells in positive lesions did not increase with progression, mean staining intensity was stronger in malignant lesions compared to benign lesions.

Ligand binding assays showed that EGFR expression in the highly metastasizing cell lines MV3 and BLM was at least 40 times higher than in the cell lines IF6, 530, M14 and Mel57 which do not or only sporadically metastasize after subcutaneous inoculation in nude mice. Although the differences between the various stages of progression are not absolute, we provide further evidence that EGFR expression increases in human melanocytic tumour progression *(J Invest Dermatol 1992; 99: 168-173).*

## INTRODUCTION

Antigens associated with tumour progression of human melanocytic cells have been identified and are detectable immunohistochemically (1-7). Different results have been reported on the expression in human melanocytic lesions of one of these antigens, the epidermal growth factor receptor (EGFR) (7,8).

The EGFR is a 170 kD transmembrane glycoprotein (9). The cytoplasmatic domain of the receptor is in structure similar to the v-erbB oncogene protein (10). Binding of epidermal growth factor (EGF) or transforming growth factor $\alpha$ to the external domain of EGFR can result in the activation of a mitogenic pathway (11). In vitro, overexpression of the human EGFR confers an EGF dependent transformed phenotype to NIH 3T3 cells (12).

Elder et al (7) reported that the EGFR shows the distribution of a progression antigen in human melanocytic lesions. However, in another series of human melanocytic lesions studied immunohistochemically by Real et al. (8) no EGFR expression was found. As these authors used different immunohistological techniques and different monoclonal antibodies (MoAb), we wondered whether this could explain the dissimilar results. Therefore, we first compared the sensitivity of four routinely applied immunohistochemical methods using a panel of six human melanoma cell lines with different levels of EGFR expression. The MoAbs used by Elder et al. (MoAb 425) (13) and by Real et al. (MoAb 225) (14) were compared with a third one, MoAb 2E9 (15), which appeared very sensitive in a pilot study. In addition, the level of EGFR expression in the cell lines was quantified using a multiple point ligand binding assay. Furthermore, EGFR-mRNA expression was determined. Based on these results the most sensitive immunohistochemical method was chosen for further study of EGFR-expression in a large series of melanocytic lesions. We provide further evidence for a differential expression of EGFR, both in human lesions of various stages of melanocytic tumour progression and in human melanoma cell lines with a different metastatic behavior in nude mice.

## MATERIALS AND METHODS

### *Cell lines*

Human melanoma cell lines used, included the following: IF6 (16), 530 (17), Mel 57 (18), M14 (19), BLM (16) and MV3 (20). Cell lines IF6, BLM and MV3 were developed in our laboratory. BLM is a subline of the BRO line (21) with a higher metastatic potential in nude mice than the parental line. Further-

more, the vulval carcinoma cell line A431 was used (22). All cell lines were grown as monolayer in culture flasks on Dulbecco's Modified Eagle's Medium supplemented with 10% Fetal Calf Serum, glutamine, penicillin G and Streptomycin. Cells were harvested by short trypsinization and processed for cytospin preparation or RNA isolation.

*Human melanocytic lesions*

Subcentral representative slices of 16 common nevocellular nevi (NN), 13 dysplastic nevi (DN), 48 primary cutaneous melanomas (PM) and 22 melanoma metastases (MM) were snap-frozen in liquid $N_2$ and stored at -70°C until use. The histopathological diagnosis was made on corresponding paraffin sections and the representativity of the samples of the frozen sections was ascertained. Primary melanomas were divided into three categories according to the Breslow thickness (23): $\leq$ 0.76 mm (PM1), between 0.76 and 3 mm (PM2), $\geq$ 3 mm (PM3) in thickness. The degree of atypia in DN was assessed (24).

*Monoclonal antibodies and immunocytochemical techniques*

MoAb included: 1) 2E9 (ascites fluid) (15), 2) 425 (tissue culture supernatant) (13), 3) 225 (1 mg/ml purified immunoglobulins)(14). All three murine MoAb recognize an antigenic determinant of the protein core of the EGFR. Cytospin preparations of the cell lines were dried, fixed in acetone and incubated for 60 minutes with MoAb 2E9 (1:25), MoAb 425 (undiluted) or MoAb 225 (1:12.5). The further procedure consisted of one of four different immunoperoxidase techniques; 1) a two-step method: incubation with peroxidase-labeled rabbit anti-mouse Ig serum (RAMPO, Dakopatts, Denmark), 2) a three-step method: incubation with RAMPO followed by incubation with peroxidase-labeled swine anti-rabbit Ig serum (Dakopatts, Denmark), 3) a PAP method: incubation with rabbit anti-mouse Ig serum (Cappel, Belgium), followed by incubation with swine anti-rabbit Ig serum (Dakopatts, Denmark) and incubation with rabbit PAP complex (Dakopatts, Denmark), 4) an avidin-biotin-complex (ABC) method (Vector Laboratories, USA). All incubations were performed at room temperature for 30-60 minutes. Between incubations sections were rinsed with PBS. Visualization of the immunoperoxidase reactions was performed with 3-amino-9-ethylcarbazole as substrate. Preparations were counterstained with hematoxylin. Titration experiments with the MoAbs were performed using method 2.

Based on the staining results of the cytospin preparations, air-dried and acetone

fixed frozen 4 μm sections of melanocytic lesions were processed using MoAb 2E9 and the three-step immunoperoxidase technique (method 2).

*Score*

The intensity of staining in the melanocytic cells was scored semiquantitatively as: -(no), ±(weak), +(moderate), ++(marked). In the cytospin preparations of the cell lines the percentages of positive cells and the average staining intensities were estimated. In the frozen sections of human melanocytic lesions the percentages of positive melanocytic cells were estimated. Lesions were regarded EGFR positive if 5% or more of the melanocytic cells in the lesion stained.

*Ligand binding assay*

Cells were harvested with a rubber policeman and homogenized by means of ultrasound bursts (MSE Soniprep-150: nominal frequency 23 kHz., amplitude 10 μm) for 10 seconds, on ice, in EGFR assay buffer (0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and 70 μg/ml Bacitracin).

The homogenates were centrifuged for 10 minutes at 800xg, 4°C, to spin down nuclei and other coarse cell fragments. The supernatants were recentrifuged for 60 minutes at 105,000xg, 4°C. The cell membrane pellets thus obtained were resuspended in 1.1 ml of EGFR assay buffer by means of ultrasound bursts. EGFR assays were performed in a manner similar to that described previously (25). To summarize: eight times 100 μl aliquots of cell membrane preparation were incubated with $^{125}$I-mouse-EGF (mEGF) tracer at concentrations ranging from 0.15 to 3.5 nM. Aspecific binding was assessed in duplicate using 1 nM $^{125}$I-mEGF and a 250-fold excess of unlabelled mEGF. Receptor-bound and free ligand were separated using hydroxyapatite. Receptor values were calculated by Scatchard analysis and expressed in fmol/mg of membrane protein.

*Northern blot analysis*

Total RNA of the cell lines was isolated by the LiCl/urea method (26). Ten μg aliquots of total RNA were glyoxylated (27) and size fractionated on a 1% agarose gel followed by blotting on hybond-N-membrane according to the procedure recommended (Amersham, UK). Hybridizations were performed as described (28). To detect EGFR mRNA the 0.8 Kb EcoRI fragment of Lambda HER A 64 was used as a probe (29). For control hybridizations on the amount of RNA a 28S rRNA probe was used. Both probes were radio-labelled using the multiprime labelling method (Amersham, UK).

## RESULTS

### *Immunocytochemistry of the cell lines*

Table I (columns A) summarizes data on the EGFR expression in the melanoma cell lines and cell line A431 as obtained by applying different MoAb and immunocytochemical staining techniques.

**Table I: Expression of EGFR-protein and EGFR-mRNA in human melanoma cell lines and cell line A431**

| Cell line | A[a] | | | | | | B | C |
|---|---|---|---|---|---|---|---|---|
| | two-step method | | | three-step method[b] | | | | |
| | 425 | 225 | 2E9 | 425 | 225 | 2E9 | | |
| IF6 | - | - | - | - | - | - | - | - |
| 530 | - | - | - | - | - | ± | 10 5 | ± |
| M14 | - | - | - | - | - | ± | 33.9 | ± |
| Mel 57 | - | - | - | - | - | - | - | - |
| BLM | - | - | + | + | + | ++ | 1436 | ++ |
| MV3 | - | - | + | + | + | ++ | 1480 | ++ |
| A431 | ND | ND | ND | ND | ND | +++ | 17271 | +++ |

[a] A EGFR expression as detected immunohistochemically using the monoclonal antibodies 425, 225 and 2E9.
Explanation of symbols; - : no staining; ± : weak staining; + : clear staining; ++ : marked staining; +++ intens staining; ND. not determined.

B EGFR expression in fmol per mg of cell membrane protein as determined with a ligand binding assay. - = not detectable (< 6 fmol/mg)

C EGFR mRNA expression determined by Northern blot analysis.
- = not detectable; ± faint-signal; ++ marked signal; +++ strong signal.

[b]: same scores were obtained using the three-step-, the PAP- or the ABC-method.

For each separate MoAb, the intensities of the staining signals were similar when comparing the three-step immumunoperoxidase technique, the PAP technique and the ABC technique. The two-step technique was less sensitive. With regard to the MoAb used at the indicated dilutions, a less sensitive or negative staining result was obtained with MoAb 425 and MoAb 225, compared with MoAb 2E9. Titration experiments showed similar staining signals for the MoAb 2E9, 425 and

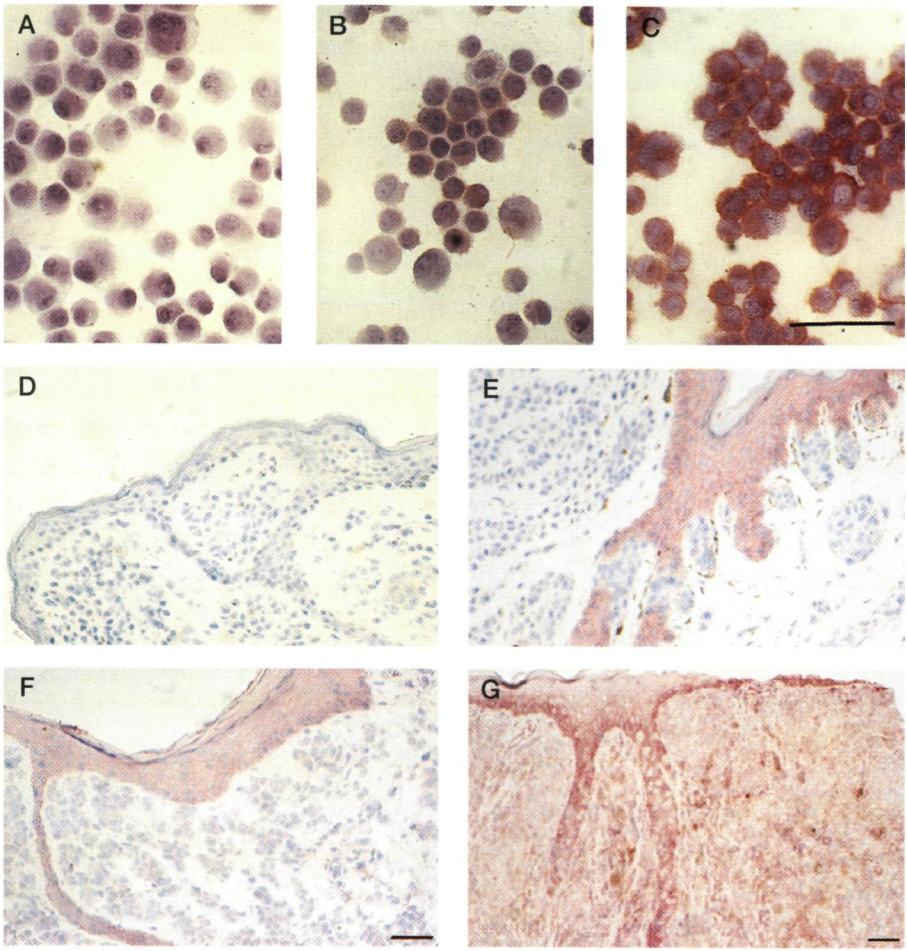


**Figure 1: EGFR expression in human melanoma cell lines and lesions.**
**A-G:** EGFR expression in human melanoma cell lines (A-C, cytospin preparations) and human melanocytic lesions (D-G, frozen sections). Immunocytochemical staining using the monoclonal antibody 2E9 and a three-step detection method. Scale bar = 50 $\mu$m. **A:** cell line IF6 showing no staining, as also found in cell line Mel 57; **B:** cell line M14 showing weak staining, as also found in cell line 530; **C:** cell line BLM showing marked staining, as also found in cell line MV3; **D:** common nevocellular nevus; negative control; the primary MoAb 2E9 was omitted; **E:** common nevocellular nevus; nevus cells show no staining, the epidermis shows a marked staining; **F:** common nevocellular nevus; nevus cells show a weak staining in a diffuse pattern, the epidermis shows a marked staining; the mean staining intensity observed in positive dysplastic nevi was similar to the staining shown here; **G:** primary cutaneous melanoma; diffuse intermediate to marked staining of melanoma cells; the mean staining intensity observed in the positive melanoma metastases was similar to the staining shown here.

225, when used diluted 1:300, undiluted and diluted 1:12.5, respectively. Using the MoAb 2E9 (1:25) and the three-step immunoperoxidase detection method cell lines IF6 and Mel 57 were negative, 530 and M14 weakly positive and BLM and MV3 markedly positive (Fig. 1A-C). The positive reference cell line A431 showed a very strong staining. In the positive melanoma cell lines 80-100% of the tumour cells stained.

*Ligand binding assay of the cell lines*

In Table I (column B) and Fig. 2 the level of EGFR expression in the cell lines, as quantified by radio-ligand binding assays is shown. Cell line A431 showed a very high expression. Cell lines MV3 and BLM showed a high, cell lines M14 and 530 a low and cell lines IF6 and Mel 57 no measurable level of expression. All the EGFR containing melanoma cell lines showed one single class of high affinity EGFR binding sites. In contrast to this, the A431 cell line, containing high levels of EGFR, showed a curvilinear Scatchard-plot (linearized in Fig. 2), indicating the presence of two EGFR binding sites with different affinity. The quantitative ligand binding assay data on EGFR expression in the cell lines correlated well with the semiquantitative immunocytochemical results obtained with the MoAb 2E9 using a three-step detection method. This correlation was not found with the MoAb 425 and the MoAb 225 (Table I).

*Northern blotting of the cell lines*

EGFR gene transcripts (10.5 and 5.8 Kb) were present in four out of six human melanoma cell lines tested [Fig. 3 and Table I (lane C)]. Cell lines BLM and MV3 showed a high (++), M14 and 530 a low (±), and IF6 and Mel 57 no detectable (-) level of EGFR-mRNA expression. Cell line A431 showed a very high EGFR mRNA expression (not shown).

The results on the EGFR expression in the cell lines, as detected with the MoAb 2E9 using the three-step technique correlated well with the EGFR-mRNA levels in the same cell lines.

*Immunohistochemistry of melanocytic lesions*

The expression of the EGFR in frozen sections of the melanocytic lesions as assessed immunohistochemically is summarized in Table IIA. For the different categories it shows the percentage of lesions in which $\geq 5\%$ of the melanocytic cells stained. As in the cell lines M14 and 530 there was a good correlation between the weak immunocytological staining, the ligand binding assay data and

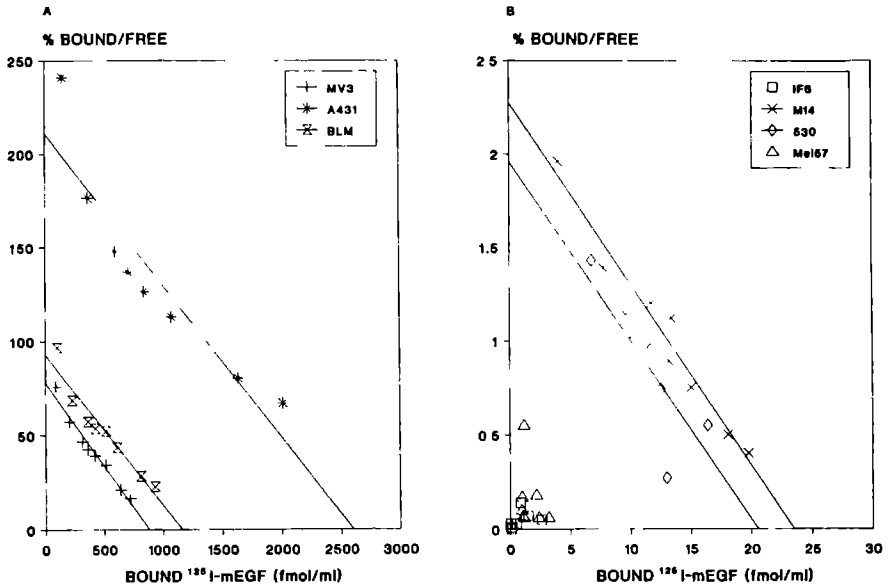


**Figure 2: EGFR expression in human melanoma cell lines.**

A and B: EGFR expression in human melanoma cell lines and cell line A431. Scatchard plot of data obtained with a ligand binding assay. The ordinate intercept depicts the level of EGFR expression in fmol/ml. Note the scale difference between A and B.

**Table II: Expression of EGFR in human melanocytic lesions[a]**

| | category[b] | | | | | |
|---|---|---|---|---|---|---|
| | NN | DN | PM1 | PM2 | PM3 | MM |
| A[c] | 19 | 61 | 87 | 85 | 95 | 91 |
| B | 45 | 67 | 56 | 36 | 42 | 60 |

[a]: EGFR expression as detected immunohistochemically (MoAb 2E9, three-step method).

[b]: Abbreviations: NN: common nevocellular nevus, DN: dysplastic nevus, PM1: primary melanoma (Breslow $\leq$0.76 mm), PM2: primary melanoma (Breslow between 0.76 mm and 3 mm), PM3: primary melanoma (Breslow $\geq$3 mm), MM: melanoma metastasis.

[c]: A: Percentage of positive lesions per category.
B: Mean of percentages of positive melanocytic cells in positive lesions per category.

the mRNA level, weak staining in the sections was regarded as relevant and taken into account. A clear difference was seen in EGFR expression, when comparing benign and malignant lesions (Table II). The percentage of positive lesions was 19 for common nevocellular nevi, 89 for primary melanomas and 91 for melanoma metastases. The dysplastic nevi hold an intermediate position (61%). In primary melanomas there was no clear increase in the number of positive lesions with increasing Breslow thickness.

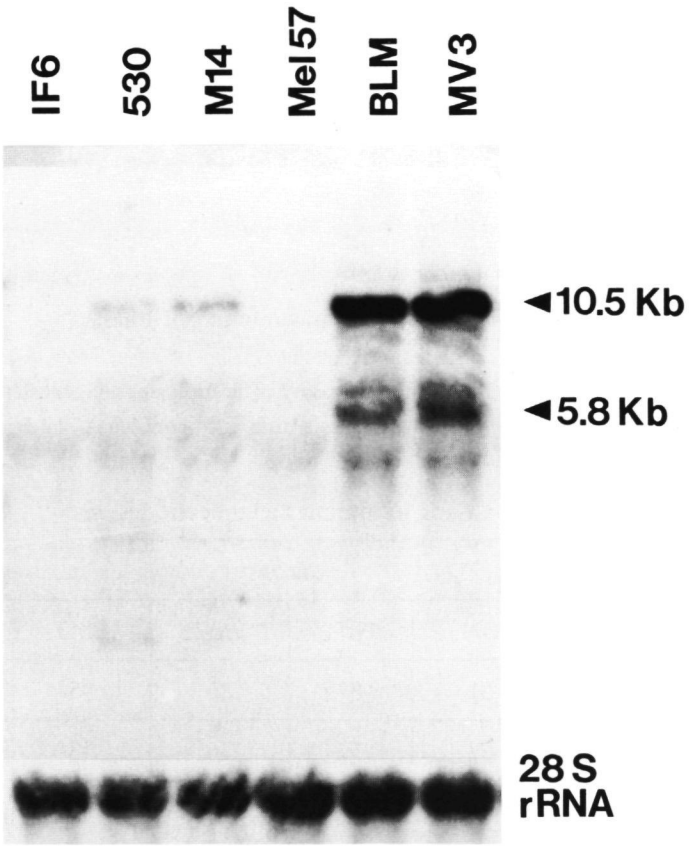


**Figure 3: EGFR mRNA expression in human melanoma cell lines.**
Northern blot analysis of EGFR mRNA expression in human melanoma cell lines using the 0.8 Kb fragment of lambda HER 64 as a probe. Two bands (10.5 and 5.8 Kb) are visible in cell lines BLM and MV3 (intense) and in cell line 530 and M14 (faint). No band could be detected with the cell lines IF6 and Mel 57. As a control on the amount of RNA, the blot was re-hybridized with a 28S rRNA probe. As molecular weight marker lambda DNA digested with endonuclease Hind III was used.

Within the group of dysplastic nevi there was no correlation between a history of dysplastic nevus syndrome or the histological degree of atypia and EGFR expression. Heterogeneity of EGFR expression was observed in most lesions. In each category of lesions the percentage of positive melanocytic cells per lesion varied (Fig. 4). With regard to this parameter there was no correlation with the stage of progression (Table IIB). Comparing the different categories, the average staining intensity in positive nevi was weak, in primary melanomas and metastatic melanomas intermediate to marked (Fig. 1D-G).

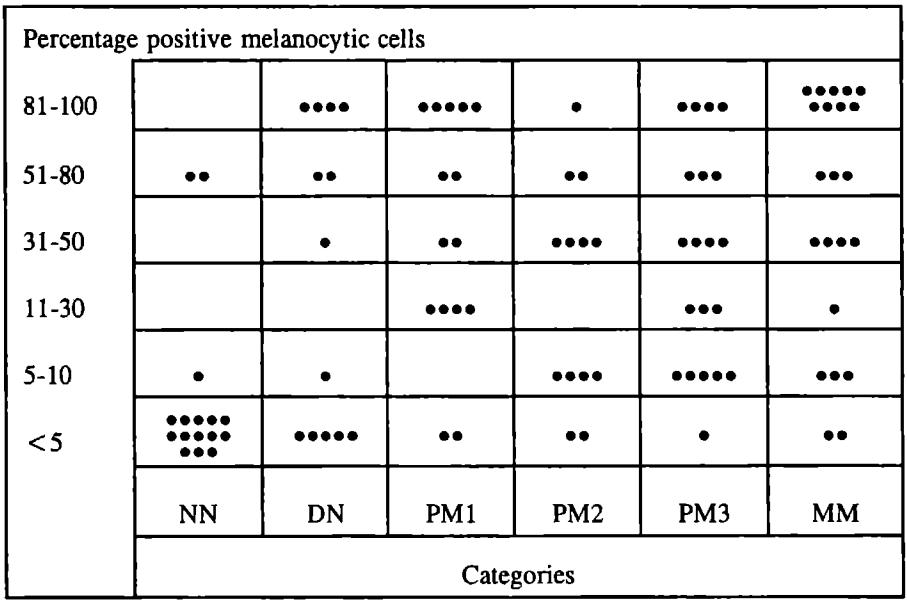


**Figure 4: Heterogeneity of EGFR expression in human melanocytic lesions.**
Diagram showing heterogeneity of EGFR expression in human melanocytic lesions, based on immunohistochemical staining using the monoclonal antibody 2E9 and a three-step detection method. Each dot represents the estimated percentage of melanocytic cells stained in one lesion. Lesions are ordered by category. NN: common nevocellular nevus; DN: dysplastic nevus; PM1: primary melanoma (Breslow $\leq$0.76 mm); PM2: primary melanoma (Breslow between 0.76 mm and 3 mm); PM3: primary melanoma (Breslow $\geq$3 mm); MM: melanoma metastasis.

## DISCUSSION

In this study we have compared the level of EGFR expression in six human melanoma cell lines using three MoAb, four immunocytochemical techniques, a

multiple point ligand binding assay and Northern blot analysis. The level of EGFR expression in cell line A431, used as a control, was very high, which is in agreement with earlier reports (8,15,29). A relatively low expression of EGFR could be detected immunocytochemically only, when MoAb 2E9 and a three-step staining method were used. Semi-quantitative data obtained with this most sensitive approach correlated well with the quantative data obtained with the ligand binding assay. Northern blotting results were also in concordance.

We quantified the differences in EGFR expression between melanoma cell lines with a different metastatic behavior in nude mice (16). Cell lines which frequently give rise to spontaneous lung metastases (BLM and MV3) had a high level of EGFR expression, whereas those with no (IF6 and 530) or only a low (M14 and Mel 57) metastatic potential had no detectable or a low level of expression. These data suggest that an increased level of EGFR expression may be relevant in melanocytic tumour progression. Supporting data, obtained with other cell types, come from studies by Di Fiore et al (12) and Velu et al (30), who found a transformed phenotype of NIH 3T3 cells after induction of EGFR overexpression by transfection experiments and from a report by Wells et al (31) who described a transformed phenotype of NR6 cells expressing a transfected non-internalizing mutant EGFR.

Because of the good correlation with the ligand binding assay results, we considered a weak immunocytochemical staining, as obtained in the cell lines 530 and M14 with MoAb 2E9 and a three-step method, as specific and relevant. This is underscored by the fact that cell lines IF6 and Mel57 were EGFR negative with both techniques.

By doing so, we found differential EGFR expression in benign, premalignant and malignant human melanocytic lesions. About 20% of common nevocellular nevi, 60% of dysplastic nevi and 90% of primary and metastatic melanoma lesions showed staining in at least 5% of the melanocytic cells. Two previous reports have dealt with EGFR expression in human melanocytic lesions (7,8). Although Real et al (8) were able to detect EGFR expression in 19 out of 36 melanoma cell lines by rosetting assay analysis, they could not detect EGFR expression in five of five primary melanoma lesions and seven of seven metastases immunohistochemically, using MoAb 225 and an ABC-procedure. Elder et al (7) detected EGFR immunohistochemically in two of eleven dysplastic nevi, in three of sixteen radial growth phase primary melanomas, in eight of nine vertical growth phase primary melanomas and in eight of ten metastases, using a PAP procedure and MoAb 425. Mature dermal nevi were found negative.

The discrepancy between our results and those of Real et al. (8) might be due to the different antibodies used, as equally sensitive immunohistochemical procedures for the detection of EGFR were applied. Our results suggest that the MoAb 225 may have a lower avidity than MoAb 2E9. A factor which may be of major importance is the fact that the two MoAb recognize different epitopes (32). For the detection of the EGFR expression in melanoma cell lines Real et al. used a highly sensitive rosetting assay and found a positive reaction in about half of the lines, which is in agreement with our results. The differences in tissue distribution of EGFR, reported by Elder et al (7) and in the present study might also be explained by the fact that different MoAb were used. In our comparance of MoAb and detection methods on a panel of melanoma cell lines, we found for the equally sensitive three-step- and PAP-procedures that the staining signal obtained using MoAb 2E9 was stronger than that of MoAb 425. In addition, the positive correlation between the staining signal obtained with MoAb 2E9, the EGFR level as determined by ligand binding analysis and the level of mRNA expressed was not found for MoAb 425. MoAb 2E9 was used as ascites fluid and the MoAb 425 as tissue culture supernatant. We assume that the concentration and the avidity of the MoAb used have influenced the staining results. Furthermore, the MoAb may recognize different epitopes (13,15). The outcome of our study of melanocytic lesions was not altered essentially when a 10% margin was applied to score a lesion as "positive". If only the lesions showing a staining intensity of + or more would be regarded relevant, thus excluding the weak staining signals, no benign and fewer premalignant lesions would be scored positive. However, we were unable to detect differential staining in horizontal growth phase primary melanomas as compared to vertical growth phase primary melanomas, as reported by Elder et al (7).

From this study we conclude that, although the differences between the various stages of progression are not absolute, we provide further evidence that EGFR expression increases in human melanocytic tumour progression.

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# CHAPTER 5

# In situ detection of supernumerary aberrations of chromosome-specific repetitive DNA targets in interphase nuclei in human melanoma cell lines and tissue sections

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**ABSTRACT**

The use of non-radioactive in situ hybridization (ISH) with chromosome specific repetitive DNA probes to study genomic changes, aneuploidy and heterogeneity during melanocytic tumour progression, relies on its applicability to non-mitotic interphase nuclei, present in cell suspensions and tissue sections. Therefore, we studied the feasibility of detecting numerical aberrations with respect to the (peri-)centromere regions of chromosomes 1 and 7 in intact nuclei of two human melanoma cell lines with different metastatic behavior in nude mice. In addition, we used paraffin sections from xenograft lesions, obtained by inoculation of these cell lines in nude mice (subcutaneous tumours and spontaneous lung metastases). Paraffin sections from the original primary cutaneous melanoma (with a subepidermal and a dermal part) and two loco-regional metastases were also studied, one of which was the source for the cell lines. These cells and tissues represent examples of materials used in different approaches to study melanocytic tumour progression.

Regarding the targeted sequences, ISH analysis showed that both cell lines were heterogeneous and aneuploid. The results correlated well with those obtained by ISH on metaphase spreads. Differences between the lines, which could not be detected by flow-cytometric or conventional karyotyping analysis, included data suggestive of a polyploid subpopulation and an extra copy of chromosome 7 in the metastasizing cell line. The polyploid population could be detected also in the paraffin sections of the corresponding subcutaneous xenografts and lung metastases in the mice. Both areas in the patients' primary melanoma could be evaluated separately and showed similar supernumerary aberrations of the chromosome specific targets. These abnormalities matched those found in both metastases.

Our results demonstrate that ISH can be used to visualize genomic abnormalities at the single cell level in melanocytic nuclei in their natural context, which makes it a promising tool in the histopathology of melanocytic lesions and in the study of melanocytic tumour progression *(J Invest Dermatol 1992; 98: 450-458)*.

## INTRODUCTION

In human melanocytic tumour progression (1), the identification of genomic changes is based on the comparison of cells from different stages. Regarding the total DNA content of tumour cells, flow-cytometric studies have highlighted the correlation between aneuploidy and tumour progression in melanocytic lesions (2), whereas Southern blotting and restriction fragment length polymorphism analysis have been used in the study of single genes (3). Cytogenetic studies in particular have directed attention to specific chromosomes of interest in malignant melanoma (for review see Ref 4). Most karyotyping studies reported are confined to melanoma metastases or metastasis derived cell lines: alterations of chromosome 1, chromosome 6 and chromosome 7 have been encountered most often in these late stages (4). Due to the low mitotic index and the limited amount of tissue available, it is difficult to obtain metaphases from early (precursor) lesions (5-11). Consequently, data on cytogenetic aberrations in subsequent stages of melanocytic tumour progression from one patient are sparse (4,6-10). Therefore, it is worthwhile to study these changes in cell preparations and tissue sections. Furthermore, the latter allows the study of genomic aberrations in cells in their natural context and thus enables a direct in situ comparison of DNA alterations and phenotypical characteristics.

Non-radioactive in situ hybridization techniques (ISH) enable the visualization of DNA targets in interphase nuclei, a method that has been called interphase cytogenetics (12). DNA probes, which hybridize to the (peri-)centromeric region of a specific chromosome (13), have been reliably used to detect numerical chromosomal aberrations in interphase nuclei in neoplasms (14-24). The application of ISH to tissue sections (25), provides a tool to link cytogenetic to morphological characteristics (19,23,24).

In this paper we examine the feasibility of applying ISH to melanocytic interphase nuclei, in order to trace and compare heterogeneity and numerical changes with respect to centromere regions of chromosomes 1 and 7. First, we used two human melanoma cell lines, derived from one lymph node metastasis, with different metastatic behavior in nude mice (26, 27). In addition, paraffin sections from corresponding xenograft lesions in mice (subcutaneous tumours and spontaneous lung metastases) were studied. Finally, we applied ISH to paraffin sections from the patients' primary cutaneous melanoma and the loco-regional lymphnode metastases. These cells and tissues represent examples of the materials often used to study melanocytic tumour progression. As we concentrated in this study on the feasibility of detecting numerical changes, we focussed

on chromosome 1 and 7 because, in malignant melanoma, numerical changes of these chromosomes are most frequently reported (4).

## MATERIALS AND METHODS

### *Clinical data and tissue specimens*

A pigmented cutaneous nodule was removed from the chin of a 76 year-old male. Conventional histopathological examination revealed a primary cutaneous malignant melanoma of nodular type, Clark level IV, Breslow thickness 6 mm. After five months, lymph node metastases were found in the homolateral neck. A fragment from one metastasis was used for subcutaneous implantation in nude mice, while other fragments were snap-frozen and stored at -70°C. The remainder of the specimen was processed for routine histology, i.e., formalin fixation and paraffin embedding. The patient died of pulmonary metastases 15 months after diagnosis.

### *Cell lines MV1 and MV3*

Parts of one lymph node metastasis were implanted subcutaneously into nude mice. Cell line MV1 was established from part of the subcutaneous outgrowth of the first passage. Cell line MV3 was isolated from part of the outgrowth of the third passage. Both cell lines were cultured as described for MV3 (26,27). Cell lines MV1 and MV3 consistently give rise to local tumours after subcutaneous inoculation in nude mice. Mice inoculated with MV3 show spontaneous lung metastases in 90% of cases, compared to 10% after inoculation with MV1. The subcutaneous tumours and the corresponding spontaneous lung metastases were in part processed for routine histology and in part snap-frozen. To prepare ethanol fixed suspensions of the cell lines, we trypsinized cultured cells, washed them with phosphate-buffered saline (PBS), resuspended in ethanol 70% (-20°C), and stored at -30°C. As a control, an ethanol-fixed suspension of cultured normal melanocytes derived from normal human foreskin was used.

### *Flow-cytometric and karyotypic analysis of the cell lines*

To determine the cellular DNA content, we used suspensions of trypsinized cultured cells of MV1 and MV3 for flow-cytometric analysis, as described (28). Chicken red blood cells and human lymphocytes were used as standard control cells.

For karyotyping, subconfluent tumour cell cultures of MV1 and MV3 were

incubated with colchicine (1$\mu$g/ml) for 90 minutes, trypsinized, subsequently treated with a pre-warmed (37°C) hypotonic 0.075 M KCl solution for 10 minutes, and finally fixed with a mixture of methanol and glacial acetic acid (3:1). Air-dried slides were prepared according to routine cytogenetic procedures. Fifty metaphases were photographed after conventional Giemsa staining and chromosome numbers were counted from the photographs. Ten mitoses were studied in more detail after G-banding of the chromosomes. Three cells were fully karyotyped. At least 5 metaphases from both cell lines were studied after Q-banding and subsequent C-banding. Stored air-dried preparations were used for ISH on metaphases, using the same method as described below for ISH on cell suspensions.

***Pretreatment of ethanol-fixed cell suspensions for ISH***

Ten microliters of an ethanol suspension were dropped on a poly-L-lysine (Sigma, St. Louis, USA) coated glass slide (29), air-dried and heated for 60 min at 80°C. Cells were pretreated by enzyme digestion with pepsine (2500-3000 units per mg protein; Sigma) at a concentration of 100 $\mu$g/ml in 0.01 N HCl for 10 min at 37°C (17). After washes with $H_2O$ and PBS, post-fixation with 1% formaldehyde in PBS for 20 min at 4°C, and further washes with PBS and $H_2O$, nuclei were equilibrated at room temperature with hybridization buffer [60% formamide in 2x SSC (Standard Saline Citrate, 0.3 M NaCl, 30 mM Na-citrate), 0.05% Tween, pH 7.0.].

***Pretreatment of paraffin sections for ISH***

Six-micrometer paraffin sections were floated on water (40°C) and mounted on glutaraldehyde-activated poly-L-lysine coated glass slides (29). Sections were air-dried and heated for 16 hours at 56°C, dewaxed in xylene (3x10 min) and rinsed in methanol (100%, 2x5 min). Endogenous peroxidase activity was blocked in methanol/1% $H_2O_2$ for 30 min, followed by rinsing with methanol (2x10 min), all at room temperature. Sections were air-dried and nuclei were, prior to enzyme digestion, pretreated either by incubation with 1 M NaSCN at 80°C for 10 min and two subsequent washes with $H_2O$ (5 min) or by incubation with $H_2O$ for 20 min at 37°C (23,24).

Enzyme digestion was performed with pepsine (as described above) at a concentration of 4 mg/ml in 0.2 N HCl at 37°C. Digestion was "tuned" to obtain an optimal balance between morphology preservation and ISH signal. The digestion time ranged from 5-60 minutes. After pepsine treatment, slides were dip-washed

in $H_2O$, dehydrated with ethanol and heated for 30 min at 80°C.

*Probes*

The satellite III DNA probe for chromosome 1 (pUC 1.77) recognizes a tandem repeat of 1.77 Kb in the (peri-)centromere region of chromosome 1 (30). The alphoid probe for chromosome 7 (p7dl) recognizes tandem repeats in the centromere region of chromosome 7 (31).

Biotinylation of the probes was performed using Bio-11-dUTP (Sigma, St. Louis, USA)(32,33) in a nick translation reaction as recommended by the supplier (BRL, Gaithersburg, USA).

*Conditions of ISH*

Hybridization conditions of high stringency were applied (17-19). Five to fifteen microliters of the hybridization mixture (2-6 ng chromosome-specific biotinylated probe DNA, 50ng herring sperm DNA and 50 ng baker's yeast RNA per 1$\mu$l hybridization buffer containing 10% dextran sulphate) was added to the nuclei/metaphases on the slide under a coverslip and sealed with rubber cement.

Simultaneous denaturation was performed on a heating plate: 3 min at 70°C for the metaphases and the isolated cells from the suspensions, and 10 min at 80°C for paraffin sections. Hybridization was performed overnight at 37°C. Post-hybridization washes were done with hybridization buffer at 42°C (2x 5 min).

*Visualization of hybrids*

Based on procedures described before (21,34), after the post-hybridization washes, slides with cells/metaphases were subsequently washed with 2x SSC (pH 7.0, 2x5 min at 42°C) and 4x SSC/0.05% Tween (pH 7.0, 1x5 min at room-temperature), followed by pre-incubation with 5% non-fat dried milk in 4x SSC/0.05% Tween (pH 7.0, 10 min at room temperature). Biotin-labelled DNA was detected by incubation with FITC-conjugated-avidin (Vector, Burlingame, USA; 1:500 in 4xSSC/ 0.05% Tween, pH 7.0) for 20 min at 37°C. Signal amplification was obtained by incubation with biotinylated goat-anti-avidin serum (Vector, Burlingame, USA; 1:100 in 4xSSC, 0.05% Tween, pH 7.0) for 20 min at 37°C followed by a second incubation with FITC-conjugated avidin. Finally, after rinsing with PBS and $H_2O$, preparations were dehydrated with alcohol and mounted with PBS/glycerol (1:9 v/v) containing 2.3% 1,4-di-azobicyclo-(2, 2,2)octane (DABCO, Merck, Darmstadt, Germany) and 0.05 % 4,6-diamidine-2-phenylindole dihydrochloride (DAPI, Boehringer Mannheim, Mannheim,

Germany).

After the post-hybridization washes, paraffin sections were washed with PBS/0.05% Tween (2x5 min at room temperature) and pre-incubated with normal rabbit serum (NRS, 2% in PBS/0.05% Tween) for 10 min at room temperature. Slides were then incubated with mouse-anti-biotin serum (Dakopatts, Glostrup, Denmark; 1:100 in PBS/2%NRS/0.05% Tween) for 45 min at 37°C. After washes with PBS/0.05% Tween (2x5 min at room temperature) slides were incubated with peroxidase-labelled rabbit-anti-mouse serum (Dakopatts, Glostrup, Denmark; 1:100 in PBS/2%NRS/0.05% Tween) for 45 min at 37°C. After washes with PBS (3x5 min) visualization of the probe was performed using di-amino-benzidine (DAB, Sigma, St. Louis, USA; 0.5 mg/ml in PBS/0.1M imidazol) as the substrate in a peroxidase reaction. Preparations were counterstained with hematoxilin solution (Merck).

Evaluation of signals in the cells and sections was performed as described (14,16,19,23).

## RESULTS

### *Flow-cytometric and karyotypic analysis of the cell lines*

The analysis of the total DNA content of MV1 and MV3 cells by flow-cytometric analysis of propidium iodide stained nuclei showed aneuploidy in both cell lines with a DNA-index (DI) in the triploid region (DI-MV1=1.43; DI-MV3=1.46). No tetraploid or polyploid subpopulation could be detected (Fig. 1A, C). Karyotyping of the cell lines with respect to chromosome counts revealed an over-all aneuploidy, resembling a hypotriploidy in both MV1 and MV3. The chromosome number varied from 53 - 69 (X= 64.32 $\pm$ SD 2.49) for MV1 and from 45 - 74 (X= 57.08 $\pm$ SD 4.99) for MV3. Structural chromosomal abnormalities were frequent in both cell lines and several unidentified marker-chromosomes were present (Figs. 2 and 3). In MV1 the numbers of chromosome 1 and 7 centromere regions, which could be identified in the three fully karyotyped cells, were 3, 3, 3 and 3, 3, 3, respectively. For chromosome 1 this was confirmed by the C-banding experiments. ISH on metaphase-spreads using probes for the centromere region of chromosomes 1 and 7 revealed a major population with three spots and a smaller population with two spots for these chromosomes, both in the metaphases and in the interphase nuclei on the same slides (Fig. 4A, B).

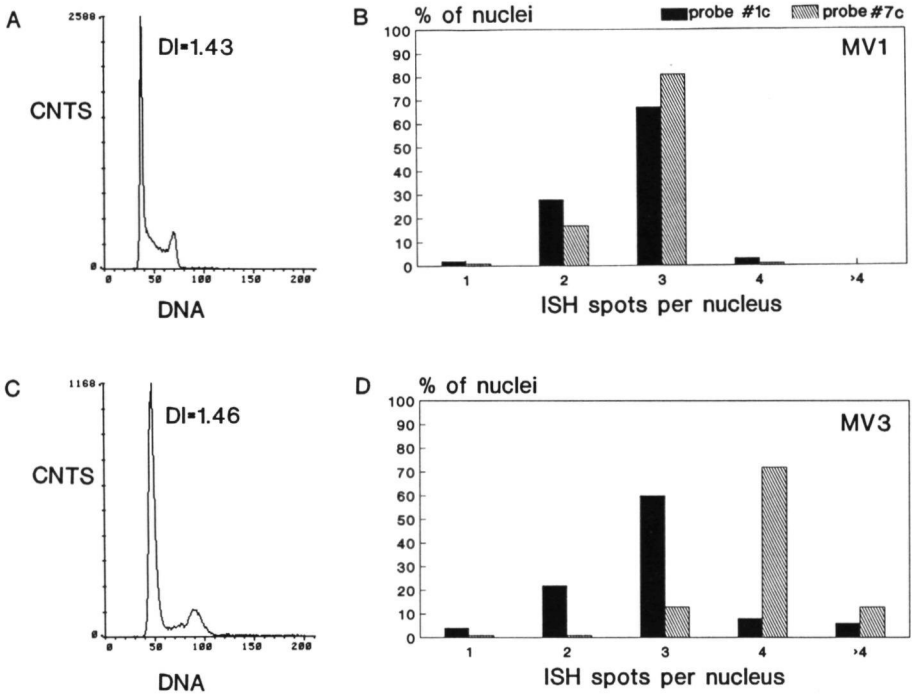


**Figure 1: Analysis of cell lines MV1 and MV3 by flow-cytometry and ISH**

**(A, C):** DNA histograms of MV1 cells (**A**) and MV3 cells (**C**) that were stained with propidium iodide and analyzed by flowcytometry. Chicken red blood cells and human lymphocytes were used as standard control. The cell lines showed a similar DNA index. DI, DNA index; CNTS, counts.

**(B, D):** Frequency distribution in the cell lines MV1 (**B**) and MV3 (**D**) of nuclei with the indicated number of hybridization spots, obtained by ISH using chromosome specific (peri-)centromere probes. Black bars, percentages obtained with pUC 1.77, the chromosome 1 specific probe (#1c); hatched bars, percentages obtained with p7d1, the chromosome 7 specific probe (#7c). 400 nuclei were counted for each evaluation. Differences between the cell lines are described in the text.

In the 3 fully karyotyped MV3 cells, the number of chromosome 1 centromere regions, which could be identified readily was 2, 2, and 3. These cells contained a dicentric chromosome 1, which also appeared in the C-banding as a dicentric chromosome 1 marker with one centromere of unknown origin (Fig. 4C, D). ISH on metaphases showed that this chromosome contained two chromosome 1

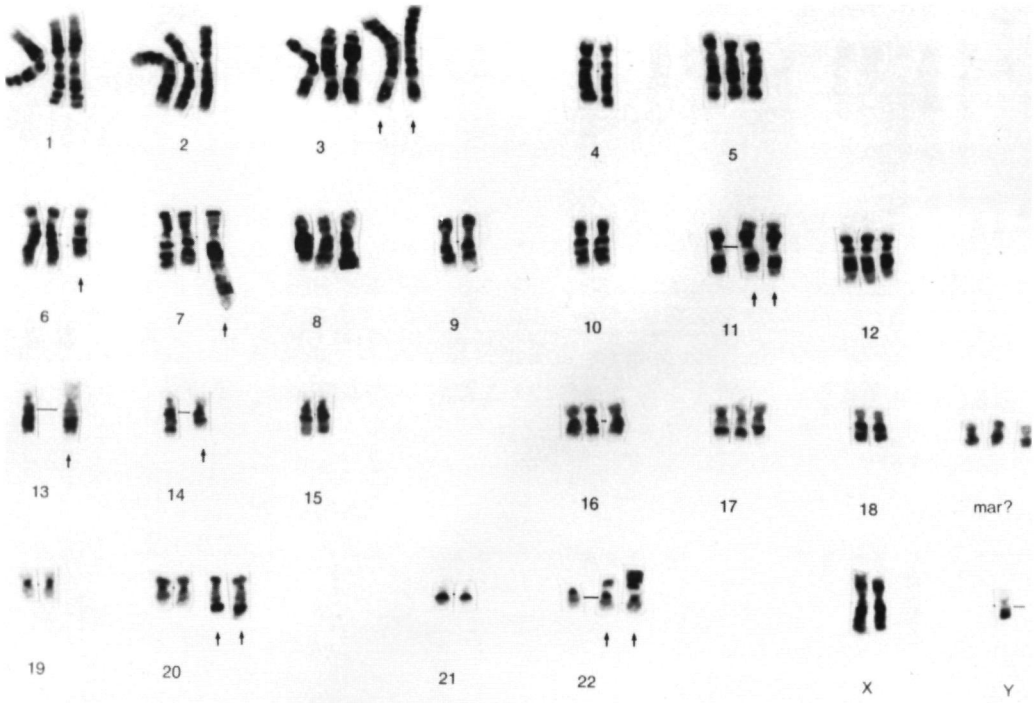


**Figure 2: Karyotype of cell line MV1**

In all cells many clonal marker-chromosomes were detected involving chromosomes 3, 6, 7, 11, 13, 14, 20 and 22 (↑). Furthermore, in every cell several unidentified marker-chromosomes were present (mar).

centromere regions (Fig. 4E, F), and that the major fraction of the metaphases had three chromosome 1 centromere regions. For chromosome 7 in MV3, ISH on metaphase preparations revealed the largest population to contain four spots, both in the metaphase spreads and in the present interphase nuclei. Populations with three, or more than four spots were also present. Some cells with two spots were seen. By G-banding, only two identifiable chromosome 7 centromere regions in the three fully karyotyped cells could be detected. Many unidentified marker-chromosomes were seen (Fig. 3).

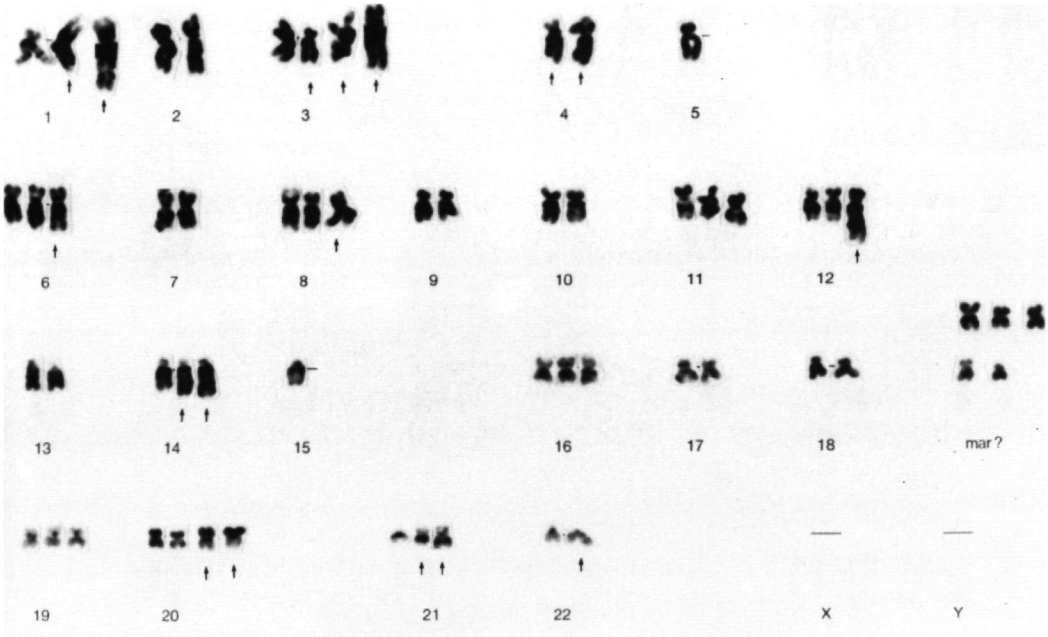


**Figure 3: Karyotype of cell line MV3**

The karyotype of MV3 showed a large complexity and variability. Many (unidentified) marker-chromosomes were present in every cell (mar, ↑). In the analyzed cells no sex chromosomes were recognized.

***ISH on cell suspensions***

In contrast to the flow-cytometric data, where the DNA histogram showed a similar DNA index for apparently homogeneous cell populations in both lines, ISH analyses with chromosome-specific probes for the centromere regions of chromosome 1 or 7, showed that there were marked differences between the cell lines MV1 and MV3 (Fig. 1B, D; Table I). Both aneuploid cell lines were heterogeneous as they showed different cell populations. This is reflected by the different number of ISH spots in the nuclei of each line (Fig. 5A, B; Table I).

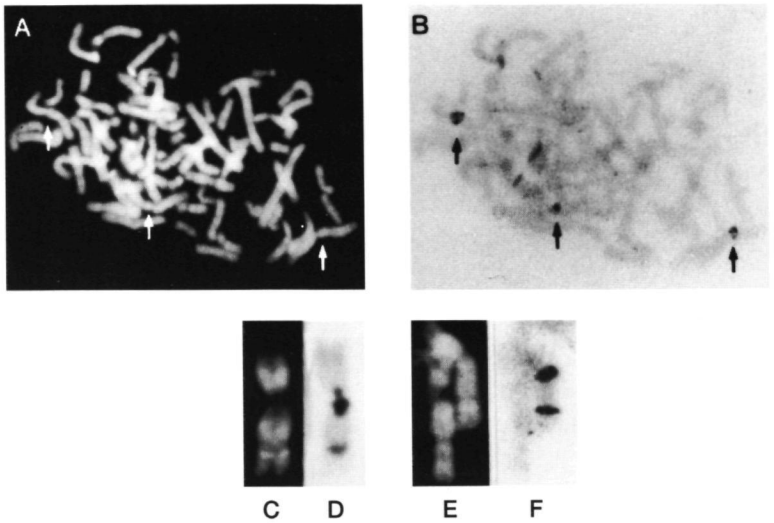


**Figure 4: ISH on metaphase chromosomes**

**(A, B):** Example of ISH on a metaphase-spread of cell line MV1, using probe pUC 1.77, hybridizing to the peri-centromere region of chromosome 1. **A:** DAPI- and **B:** FITC-image of a metaphase, showing hybridization of the probe to three regions. **(C, D, E, F):** Derivative dicentric chromosome 1 as present in all studied cells of MV3. **C:** Q-banding and **D:** C-banding of an example of this marker chromosome, **E:** DAPI- and **F:** FITC-image of two chromosomes, one of which shows two spots after hybridization with pUC 1.77.

In MV3, we detected an imbalance between the numbers of ISH spots obtained with probes specific for either chromosome 1 or 7. In the case of chromosome 1 the major population showed three spots per nucleus, in contrast to the chromosome 7 centromere probe, where the major population contained four spots per nucleus. Thirteen percent of the nuclei of the latter contained more than four, ranging from 5-11 (Figs. 1D, 5C-F). The overall picture for MV3 showed four or more spots for chromosome 7 in most nuclei, but showed three or less for chromosome 1. A subpopulation with more than four spots, as detected in MV3 for chromosomes 1 and 7, could not be traced in MV1 (Fig. 1B). In MV1 the major population for both chromosomes 1 and 7 showed three spots per nucleus. Nuclei with four spots for either chromosome 1 or 7 were rare in MV1. ISH on cell suspensions of cultured normal human melanocytes, which were used as a control, showed two spots for the chromosome 1 and 7 centromere sequences in at least 95% of the nuclei. No nuclei with more than two spots were seen.

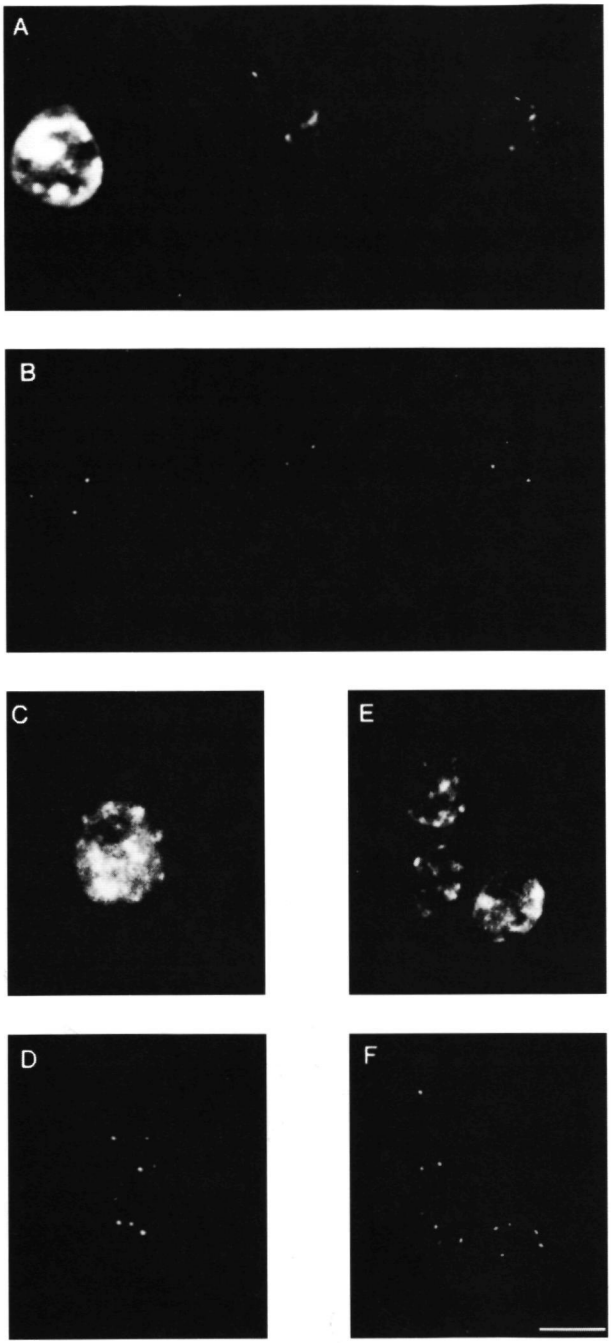


**Figure 5: In situ hybridization on cell suspension of cell line MV3. Bar, 20 $\mu$m**

**(A, C, E):** Visualization of nuclei in cell line MV3 by counterstaining of the total DNA with DAPI. **(B, E, F):** Same nuclei as in a, c and e. Visualization of the centromere region of chromosome 7, using probe p7d1. **(A, B):** Three nuclei with a different number of spots per nucleus (four, two, three), showing heterogeneity and aneuploidy. **(C, D):** Large nucleus showing eight ISH spots. **(E, F):** Two nuclei with four spots, as found in the major fraction of the nuclei in MV3 for chromosome 7, and one nucleus with five spots.

*ISH on paraffin sections*

We applied ISH to paraffin sections, in order to see whether we could detect numerical aberrations, aneuploidy and heterogeneity with regard to the chromosome 1 and 7 targets in the subcutaneous and lung lesions of the nude mice and in the primary tumour and metastases of the patient.

Using the procedures described in Materials and Methods we were able to obtain evaluable data from all tissues studied (Table I, Fig. 6A-E). As expected, no signals were observed in murine nuclei (Fig. 6D). In order to obtain optimal results, a well-tuned pretreatment prior to ISH was crucial. Compared to the cell lines, optimalization was far less easy for the paraffin sections. This is illustrated by the range of pretreatments needed, which varied from 5 minutes of enzyme digestion, without prior NaSCN incubation, to 60 minutes pepsine treatment after NaSCN pre-treatment. This reflects interlesional variability in the accessibility of the DNA targets. The need for pepsine digestion also varied within one section. As an example, the subepidermal part of the human primary melanoma was much less resistent to enzyme digestion than the compact deeper part. In general, as reported for other tissues (23,24), incubation with NaSCN shortened the digestion time with pepsine, which resulted in a more preserved morphology. In this way a better balance between ISH signal and loss of morphology was achieved. Nuclei in the paraffin sections could be evaluated with regard to the number of ISH spots in their normal architectural context (Fig. 6). Areas of interest in the sections could easily be selected. Although the melanocytic cells in general contained little melanin pigment, there was no problem in differentiating between the DAB-ISH signals in the nuclei and pigment in remaining parts of the cytoplasma.

*Xenograft lesions in nude mice*

In all the melanocytic tumour lesions in the mice, nuclei showed varying numbers of ISH spots for the chromosomes 1 and 7 targets (Table I, Fig. 6A-D). A comparison of the subcutaneous tumours of MV1 and MV3 showed the presence of a population of nuclei with more than four spots, for both the chromosome 1 and 7 probe in MV3 tumours in contrast to MV1 tumours, to be consistent with the spot distribution in the cell lines. In the MV3 tumours for chromosome 7 there was a trend towards a larger fraction of nuclei with a smaller number of spots in the subcutaneous tumour compared with cell line MV3; the imbalance between chromosomes 1 and 7 for MV3, observed in the cell line MV3, could not be found in the corresponding subcutaneous lesions in the mice. In contrast,

**Table I: Relative frequency of nuclei with indicated number of spots per nucleus, obtained by ISH on melanocytic lesions and cell lines[ab]**

| | Number of spots per nucleus | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | >4 |
| A. ISH with chromosome #1 specific probe (pUC 1.77)[c] | | | | | |
| PM[d] | ++ | +++ | +++ | ++ | + |
| MM[e] | ++ | +++ | +++ | ++ | + |
| MV1*[f] | ± | +++ | ++++ | + | - |
| EG1[g] | ++ | +++ | +++ | + | - |
| MV3*[f] | ± | +++ | ++++ | + | + |
| XG3[g] | + | +++ | +++ | ++ | ++ |
| LXG3[h] | ++ | +++ | +++ | ++ | + |
| B. ISH with chromosome 7 specific probe (p7d1)[c] | | | | | |
| PM | +++ | ++++ | ++ | + | - |
| MM | +++ | ++++ | ++ | + | - |
| MV1* | ± | ++ | ++++ | ± | - |
| XG1 | +++ | +++ | +++ | + | - |
| MV3* | ± | ± | ++ | ++++ | ++ |
| XG3 | ++ | ++ | +++ | ++ | ++ |
| LXG3 | + | +++ | +++ | ++ | + |

[a] The materials studied included cell lines (*) and paraffin sections.

[b] 95% or more of cultured melanocytes showed 2 spots for the chromosomes 1 and 7 centromere region. The majority of keratinocytes in paraffin sections of normal skin showed 2 spots. No nuclei with more than 2 spots were seen.

[c] - = 0%, ± = 0-2%, + = 3-10%, ++ = 11-20%, +++ = 21-60%, ++++ = 61-100%.

[d] PM = human primary melanoma

[e] MM = human melanoma metastases

[f] MV1 and MV3 = cell lines MV1 and MV3

[g] XG1 and XG3 = "xenografts" after subcutaneous inoculation with MV1 or MV3 in nude mice

[h] LXG3 = lung metastases in nude mice inoculated with MV3 cells

for MV3 (chromosome 1) and MV1 (chromosome 7) a proportionally larger population of nuclei with four spots or more was observed in the subcutaneous tumours than in the corresponding cell lines.

The spontaneous lung metastases of mice subcutaneously inoculated with MV3

cells, showed a similar distribution in the relative frequency of ISH spots as the "primary" tumour, both for chromosome 1 and 7 (Table I). Micrometastases could be detected by the presence of ISH spots in nuclei (Fig. 6C). Furthermore, tumour cell emboli of multiple and single melanoma cells were seen in microvascular lumina (Fig. 6D).

***Human primary melanoma lesion and metastases***

Within the primary melanoma two different areas (a micronodular subepidermal area and a deeper larger nodule) could be evaluated. Both areas were aneuploid. Subpopulations of nuclei with a given spot number were present in both areas. Comparing the two areas, no differences in the relative fraction of these nuclei could be detected. In general, nuclei with a higher spot number were larger than those with fewer ISH signals. Nuclei were found with one, two, three and four spots per nucleus for the chromosome 1 and 7 targets, whereas for chromosome 1 nuclei with five spots were observed as well (Table I, Fig 6E). The presence of these nuclei with five ISH spots contrasts with the absence of such a population in the cell line MV1. The relative frequency of nuclei with a given number of spots differed for the chromosomes 1 and 7 sequences; nuclei with three spots or more were more frequent in the case of chromosome 1. Furthermore, nuclei with more than four spots were not detected for chromosome 7. This imbalance was not present in cell line MV1 and was reversed for MV3. Although with those pretreatments which gave optimal results for the tumour cells, only a few nuclei of the overlying epidermis in this lesion were evaluable, these nuclei consistently showed no more than two spots. Control experiments on 6 $\mu$m paraffin and frozen sections of normal skin showed two spots for both probes in the majority of the epidermal nuclei. No nuclei with more than two spots were seen.

Two different neck lymph node metastases, one of which was used to make the cell lines, were evaluated and showed aneuploidy (Table I). The same relative fractions of nuclei with a given spot number were observed in these two lesions. This distribution also matched that of the primary melanoma. Thus, comparing the primary melanoma and the two metastases with regard to the nuclei with more than four spots, this subpopulation was present in the case of chromosome 1 and absent for chromosome 7 in all three lesions.

## DISCUSSION

Melanocytic tumour progression can be studied by comparing melanoma cell lines with different biological behavior or by comparing (parts of) tissues with a different morphology (1). Here, we report on the feasibility of detecting chromosome specific repetitive DNA targets of chromosomes 1 and 7 in intact nuclei, using non-radioactive in situ hybridization. First, nuclei from cell suspensions of two melanoma cell lines, derived from the same patient lesion were compared. These lines show different metastatic behavior in nude mice, when inoculated subcutaneously. Regarding the total DNA content, flow-cytometric analysis of these cell lines showed apparently homogeneous cell populations in both lines with no differences in ploidy. At the single cell level, however, both cell lines showed different subpopulations, when ISH was applied using probes which hybridize to repetitive sequences in the (peri-)centromere regions of either chromosome 1 or 7. We detected an imbalance in the presence of these sequences, as reflected by the number of ISH spots in cell suspensions of MV3, but not in those of MV1. Furthermore, only MV3 showed a subpopulation with more than four ISH spots.

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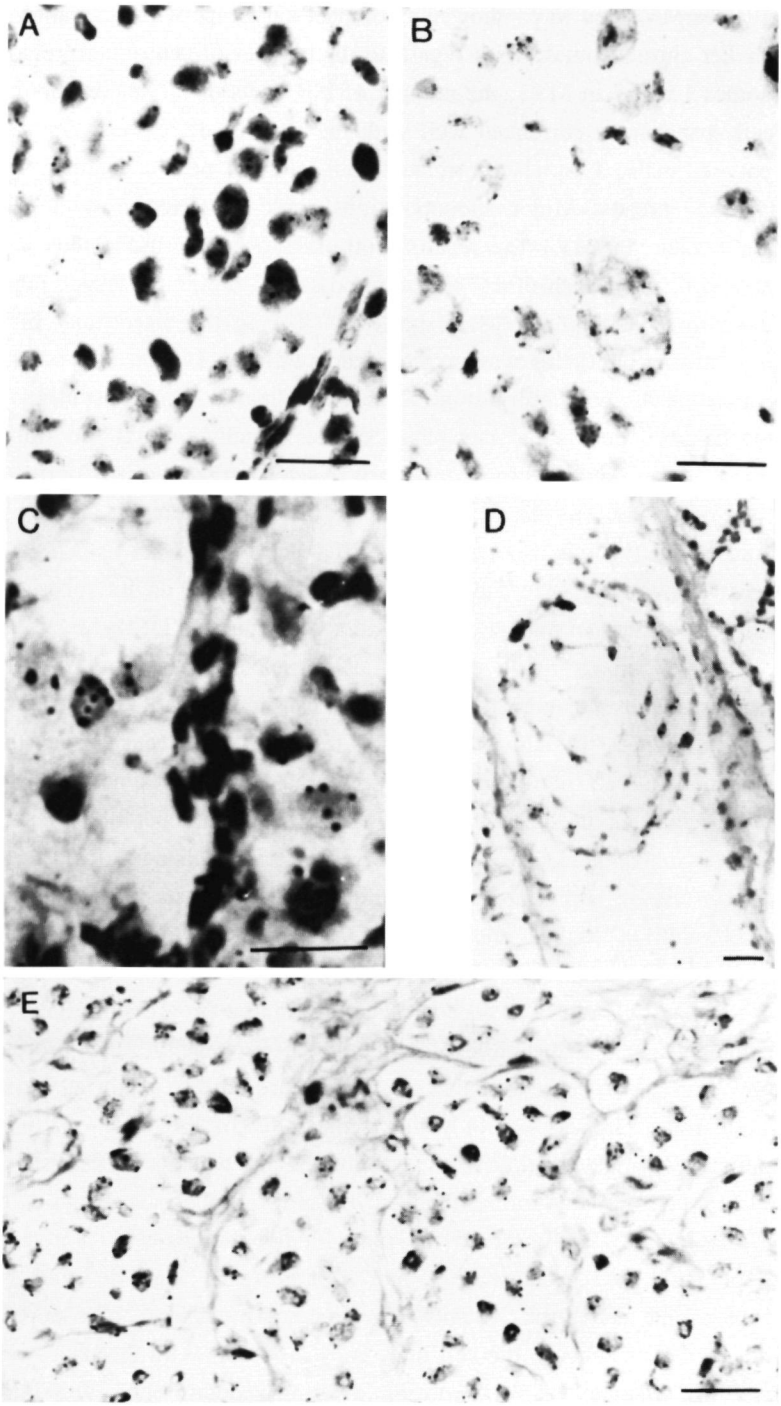
**Figure 6 (next page): ISH on 6 $\mu$m paraffin sections of melanocytic tissues**

The number of centromere regions per nucleus, for the chromosome under investigation, can be evaluated by counting the number of DAB spots per nucleus. Counterstaining of the nuclei with hematoxylin. Photographic documentation is complicated by focussing problems. Per section 200 nuclei were counted. Bar, 20 $\mu$m.

**A:** ISH with a chromosome 1 specific probe on a section of a subcutaneous tumour in a nude mouse after inoculation of cell line MV1 . The major fraction consists of nuclei with two or three spots per nucleus; **B:** ISH with a chromosome 1 specific probe on a section of a subcutaneous tumour in a nude mouse inoculated with cell line MV3. As in the suspensions, giant nuclei with a large number of spots could be detected. **C:** ISH with a chromosome 7 specific probe on a section of an area in the lungs of a nude mouse, inoculated subcutaneously with cell line MV3, to which human tumour cells have metastasized. Human cells with five spots are visible, whereas the murine lung tissue is negative; **D:** ISH with a chromosome 7 specific probe on a section showing a murine pulmonal blood vessel containing a tumour embolus of human tumour cells in a nude mouse inoculated subcutaneously with cell line MV3; **E:** ISH with a chromosome 1 specific probe on a section of the subepidermal part of the primary cutaneous melanoma. The typical tissue architecture of the tumour is preserved. Aneuploidy is visualized. Most nuclei showed two or three DAB spots per nucleus.


A
B
C
D
E

Both cell lines MV1 and MV3 showed a complex karyotype with several (unidentified) marker-chromosomes. With regard to the number of centromere regions of chromosomes 1 and 7 in MV1, the number of ISH spots of the major populations in the cell suspensions correlated well with the number of regions identified in the karyotyped cells. They also correlated well with the results obtained by ISH on metaphase spreads. Minor subpopulations could be detected with ISH on interphase nuclei. In MV3 the results from ISH on cell suspensions and on metaphases confirmed each other. For chromosome 1 and 7 the major populations showed three and four spots, respectively. Taking the dicentricity of chromosome 1 into account, this was in accordance with the G- and C-banding data for chromosome 1. For chromosome 7 aberrant (marker-)chromosomes, which could not be identified in the metaphase spreads, might harbour the "missing" chromosome 7 centromere regions. In theory, a selection caused by differences in the mitotic index during the preparation of metaphase spreads between subpopulations in MV3 could also explain the difference.

Compared to flow-cytometric and karyotypic analyses, ISH on interphase nuclei allows the assessment of numerical aberrations of specific DNA targets at the single-cell level in a large number of unselected nuclei. Aneuploidy, heterogeneity, major and minor subpopulations can be detected. The detection of numerical differences in situ with regard to specific sequences may reflect differing copy numbers of (parts of) the investigated chromosomes and enables chromosome counting in non-mitotic cells (21,22). The number of ISH spots in the major fraction of nuclei for the chromosome 7 target changed from three, in the non-metastasizing cell line MV1, to four in the metastasizing MV3. Subpopulations with a higher spot number appeared in MV3 for both the chromosome 1 and 7 sequences. The process of polyploidization, of which these observations may be indicative, was correlated to tumour progression (35).

In melanoma research aneuploidy, genotypic heterogeneity, the process of polyploidization and specific genomic aberrations are hard to study at the single cell level in intact tissues. To study the applicability of ISH to formalin-fixed, paraffin-embedded melanocytic tissues, we investigated whether differences could be traced between the xenografts, obtained by subcutaneous inoculation of either MV1 or MV3 cells. We observed the same subpopulations with a given spot number for both the chromosome 1 and 7 targets, as were detected in the suspensions of the cell lines. Notably, the presence or absence of a population with more than four spots in the cell lines correlated well with the data from the xenografts. We assume that these aneuploid lesions contain cells with different

numbers of ISH spots per nucleus, which would make them heterogeneous. The observation that small clusters of nuclei with different spots numbers were seen, supports this view. However, the different sizes of the nuclei and the effect of cutting through nuclei may lead to an underestimation of the number of spots. Thus, all nuclei may, in theory, contain the maximum number of spots detected (19,23). Regarding the lung metastases of mice inoculated with MV3, we could detect tumour cell emboli in the pulmonal vessels and micrometastases in lung parenchyma, based on the presence of ISH signals in nuclei in their natural context. Nuclei with varying numbers of ISH spots were seen in a distribution similar to that in the primary subcutaneous xenograft. As was expected for the human specific probes, no hybridization signals were seen in the murine cells. This is useful in studies, where questions may rise as to the origin of cell populations, i.e. human cells in laboratory animals (26, 27).

Further, we studied paraffin sections of the original patients' primary melanoma and two lymph node metastases. It is important for the possible detection of cytogenetic changes in complex melanocytic lesions harboring different stages of progression, that we could compare ISH data from two different areas in one section of the primary melanoma, based on the proper preservation of the morphology. Thus, ISH on tissue sections has the advantage that single tumour cells can be studied in their histological context with respect to numerical chromosomal abnormalities. In both the subepidermal and the deeper intradermal area of the primary melanoma we found extra (parts of) copies of chromosomes 1 and 7. There was an imbalance between the two chromosomes studied regarding the presence of a population of nuclei with more than four ISH spots. Similar changes have been reported in karyotyping studies. Reports from Balaban et al. and Parmiter et al. on primary cutaneous melanomas revealed abnormalities of chromosome 1 and 7 in most cases, in which histologically both a radial growth phase (RGP) and a vertical growth phase (VGP) component were present, but which could not be studied separately (6-8). No aberrations of chromosomes 1 or 7 were found in the case of a RGP primary melanoma and in the RGP part of a primary melanoma, in which the RGP and VGP component could be evaluated separately (7). Cowan et al. also reported on abnormalities of chromosomes 1 and 7 in primary melanomas (10). Based on histology, our case is an advanced primary melanoma in which aberrations of chromosomes 1 and 7 may be expected taking these data from the literature into account.

We found numerical aberrations of the targeted sequences on chromosomes 1 and 7 in the patients' metastases. Numerical changes of these chromosomes have been

described consistently in the literature (4). Strikingly, the two different metastases showed the same distribution of spot numbers, which also matched that of the primary melanoma. With respect to the comparison of a primary tumour with its metastasis, similar patterns in spot distribution were also found in the MV3-derived tumours in the mice. In line with the theory of clonal dominance, proposed by Kerbel et al. (36), it is tempting to assume that the same population(s) which formed the metastases had overgrown the primary tumour site.

The study of melanocytic tumour progression in intact human tissues depends on the selection by conventional histology of (parts of) lesions with known biological behavior. By using morphological characteristics, different stages can be identified and compared. As most tissues of interest are archived in paraffin, the application of ISH to paraffin sections opens new perspectives for the study of genomic changes in melanocytic tumour biology. We were able to evaluate our paraffin material by selecting the proper pretreatment and optimal tuning. We also obtained results in 6 $\mu$m cryostat sections of the deep frozen material. However, finding the right balance, between loss of morphology and the obtaining of evaluable ISH signals, was more difficult in comparison with the paraffin sections.

From our study, we conclude that ISH can be applied to detect targeted repetitive DNA sequences at the single cell level. The results can be interpreted in terms of aneuploidy, heterogeneity and numerical aberrations of (parts of) chromosomes. Various sources of cells and tissues can be used, including archived formalin-fixed, paraffin-embedded materials, which allows for a comparison of various stages of progression. ISH on tissue sections enables direct reference to histopathological characteristics. The reliable detection of non-repetitive DNA targets in melanocytic tissue sections, allowing the in situ detection of deleted regions of chromosomes, is under study. We believe that interphase cytogenetics will broaden the perspective of integrating genomic and phenotypic characteristics of melanocytic tumour progression. Furthermore, it may serve as a diagnostic tool in histopathology.

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# CHAPTER 6

# DNA in situ hybridization as a diagnostic tool in the discrimination of melanoma and Spitz naevus

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**ABSTRACT**

As the clinical and differential diagnosis between Spitz naevus and cutaneous melanoma clinically and histologically may be very difficult, we have investigated whether DNA in situ hybridization may be helpful in resolving this problem. To this end routinely processed paraffin sections of 15 typical Spitz naevi, 15 typical nodular melanomas, and five cases originally misdiagnosed as Spitz naevus but which later metastasized and were reclassified as melanoma were analysed using a method previously described (De Wit et al, J Invest Dermatol 1992; 98: 450-458).

Microscopical semi-quantitative evaluation revealed that the number of nuclei with supernumerary aberrations of the centromere region of chromosome 1, suggestive for aneuploidy, was significantly different in Spitz naevi and nodular melanoma. The mean number of aberrant nuclei per high power field was 0.41 and 4.01, respectively ($P=0.0001$). On applying the results of the typical lesions to the equivocal, originally misdiagnosed lesions, three out of five could be identified as melanoma.

These results suggest that the application of DNA in situ hybridization may contribute to the positive identification of histologically equivocal pigmented lesions. The advantages of this technique are that it is cheap, requires little tissue, and can be applied on routinely-processed paraffin sections *(J Pathol 1994; 173: 227-233)*.

## INTRODUCTION

In the histopathology of cutaneous melanocytic proliferations the differential diagnosis between malignant and benign lesions can be very difficult; this is true especially for nodular melanoma versus Spitz naevus (1-4). Not infrequently, cases are encountered where even expert opinions differ on routinely stained histological slides. There is an evident need for additional techniques to support the histopathological diagnosis. Recently we described how DNA in situ hybridization (DNA-ISH) utilizing probes specific for repetitive DNA targets on specific chromosomal regions can be applied on sections of routinely formalin-fixed, paraffin-embedded melanocytic lesions (5). Supernumerary aberrations of chromosomal regions could thus be detected in situ; that is, by the visualization of more than two regions in one interphase nucleus, in contrast to a normal diploid cell (6). The presence of supernumerary aberrations can be interpreted as a marker of aneuploidy. According to the literature, a considerable number of the malignant melanomas are aneuploid, the percentage of aneuploid lesions increasing with tumour thickness (7). As especially thick melanoma lesions raise problems in the differential diagnosis with Spitz naevus and in view of the foregoing it is of interest that Spitz naevi have been reported as euploid (8-12). We have applied DNA-ISH on paraffin sections of routinely processed lesions to investigate whether it can be used to identify malignant melanomas by means of in situ visualization of aneuploidy. This was done on histologically typical malignant melanomas and on unequivocal Spitz naevi. More importantly, we studied a number of primary cutaneous melanocytic lesions initially misdiagnosed as Spitz naevus, but which later proved to be malignant melanomas by the subsequent development of metastases. The aim was to investigate whether DNA-ISH could be of value in the differential diagnosis of malignant melanoma and Spitz naevus.

## MATERIALS AND METHODS

### *Unequivocal cases*

Clinical and pathological data on 15 cases of Spitz naevus and on 15 cases of nodular melanoma are listed in Tables IA and IB, respectively. These histologically unequivocal cases were selected from the files of the Departments of Pathology from the University Hospital Nijmegen and the Canisius-Wilhelmina Hospital Nijmegen. As a quality control, the slides were submitted to the pathology panel of the Dutch Melanoma Working Party. The original diagnosis was confirmed in all cases.

**Table IA: Clinico-pathological data and DNA-ISH results on 15 histologically typical Spitz naevi***

| case No. | age (yrs) | sex | site | A | B | C | D |
|---|---|---|---|---|---|---|---|
| 1 | 20 | F | nose | – | 0.6 | – | 1 |
| 2 | 22 | M | ear | – | 0.4 | – | 1 |
| 3 | 4 | F | cheek | – | 0.7 | – | 1 |
| 4 | 21 | F | lower leg | – | 0.2 (1.5)** | –(+) | 2 (4) |
| 5 | 7 | F | forehead | – | 0.4 | – | 1 |
| 6 | 17 | M | knee | – | 0.4 | – | 1 |
| 7 | 24 | F | upper leg | – | 0.2 | – | 1 |
| 8 | 21 | F | abdomen | – | 0.3 | – | 1 |
| 9 | 31 | F | lower leg | – | 0.1 | – | 1 |
| 10 | 41 | M | nose | – | 0.5 | – | 1 |
| 11 | 6 | F | elbow | – | 0.9 | – | 1 |
| 12 | 5 | F | cheek | – | 0.7 | – | 1 |
| 13 | 37 | M | cheek | – | 0.0 | – | 0 |
| 14 | 14 | F | back | – | 0.1 | – | 1 |
| 15 | 5 | F | cheek | – | 0.6 | – | 2 |

*: **A, B, C, D:** DNA-ISH results:
**A:** assessment at screening: aneuploidy suspected (+); equivocal (±); aneuploidy not suspected (–); **B:** nuclei with >2 ISH spots: mean number per HPF (10 HPFs counted); **C:** nuclei with >3 ISH spots present (+) or not (–); **D:** nuclei with >2 ISH spots: maximal number observed in one HPF; **: Figures between brackets refer to counts including the giant Spitz cells (see text)

***Problematic cases***

In order to evaluate the diagnostic value of DNA-ISH in the differential diagnosis of melanoma versus Spitz naevus, 18 problematic cases were traced using the Dutch PALGA system. This computerized data system holds histological diagnoses and keywords on biopsies per individual, entered by 70 pathology laboratories in The Netherlands. Five cases were retrieved which fulfilled the following criteria: a) the original diagnosis was documented as Spitz naevus, b) melanoma metastases occurred (n=3) or there was local recurrence with evident malignant morphology (n=2), c) the differential diagnosis of the primary lesions was difficult as documented in the pathology report and also after review of the slides, d) no other primary melanoma was documented, e) there was enough representative tissue left in the paraffin block to perform the experiments. These five cases are summerized in Table II.

**Table IB: Clinico-pathological data and DNA-ISH results on 15 histologically typical nodular melanomas***

| case No. | age (yrs) | sex | site | Clark level | Breslow** | A | B | C | D |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 23 | F | buttock | III | 3.0 | + | 2.2 | – | 4 |
| 2 | 30 | M | face | IV | 2.2 | ± | 1.0 | – | 2 |
| 3 | 61 | F | flank | III | 1.8 | ± | 1.4 | – | 3 |
| 4 | 39 | M | back | III | 1.6 | – | 0.9 | – | 2 |
| 5 | 51 | F | lower leg | IV | 3.0 | – | 1.1 | – | 5 |
| 6 | 69 | F | lower leg | V | 3.9 | + | 5.2 | – | > 10 |
| 7 | 63 | F | upper leg | V | 5.7 | + | 3.1 | – | 10 |
| 8 | 47 | M | dorsum foot | IV | 7.0 | ± | 1.6 | + | 5 |
| 9 | 37 | M | back | III | 1.5 | – | 0.6 | – | 1 |
| 10 | 40 | M | back | IV | 5.5 | + | 2.5 | + | 4 |
| 11 | 64 | M | lower leg | III | 2.7 | + | 6.3 | + | > 10 |
| 12 | 62 | M | face | IV | 3.0 | + | 5.8 | – | 8 |
| 13 | 59 | F | lower leg | V | 4.0 | + | 8.2 | + | > 10 |
| 14 | 76 | M | face | IV | 6.0 | + | 11.1 | + | > 10 |
| 15 | 54 | M | back | III | 2.9 | + | 9.3 | + | > 10 |

*: **A, B, C, D:** DNA-ISH results:
**A:** assessment at screening: aneuploidy suspected (+); equivocal (±); aneuploidy not suspected (–).
**B:** nuclei with >2 ISH spots: mean number per HPF (10 HPFs counted).
**C:** nuclei with >3 ISH spots present (+) or not (–).
**D:** nuclei with >2 ISH spots: maximal number observed in one HPF.
**: Breslow thickness in millimeters.

*DNA in situ hybridization*

The DNA in situ hybridization procedure (DNA-ISH) was performed as described previously by us in detail (5), with minor modifications. In short, 4 micrometer paraffin sections were mounted on organosilane (Sigma, St. Louis, USA) coated slides, dried, heated and dewaxed. After blocking of endogenous peroxidase with $H_2O_2$ / methanol, sections were pretreated with NaSCN (80°C) or water (37°C). Enzyme digestion was performed using pepsin in HCl, followed by dehydration and heating. Differentiation in pepsin treatment time was used to obtain an optimal balance between morphology preservation and DNA-target accessibility (obtaining of ISH-signal). A biotinylated probe pUC 1.77 was used, which hybridizes to a tandem repeat of 1.77 Kb in the pericentromere region of chromosome 1 (region 1q12) (13). High stringency hybridization conditions were used as previously described (5). Visualization of the in situ hybrids was per-

**Table II: Clinico-pathological data and DNA-ISH results on five melanomas, initially misdiagnosed as Spitz naevus***

| case No. | age (yrs) | sex | site | Clark level | Breslow** | A | B | C | D |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 9 | M | dorsum foot | IV | 3.0 | + | 5.4 | − | >10 |
| 2 | 23 | M | neck | IV | 2.2 | + | 5.5 | − | 10 |
| 3 | 50 | F | back | IV | 1.8 | ± | 1.1 | − | 3 |
| 4 | 35 | F | arm | IV | 2.9 | + | 3.7 | − | 6 |
| 5 | 80 | F | cheek | V | 4.1 | − | 0.4 | − | 1 |

*: **A, B, C, D:** DNA-ISH results:
**A:** assessment at screening: aneuploidy suspected (+); equivocal (±); aneuploidy not suspected (−); **B:** nuclei with >2 ISH spots: mean number per HPF (10 HPFs counted); **C:** nuclei with >3 ISH spots present (+) or not (−); **D:** nuclei with >2 ISH spots: maximal number observed in one HPF
**: Breslow thickness in millimeters

formed using an avidin-biotin-complex method and diaminobenzidine (DAB), resulting in dark brown spots in the nuclei in the tissue sections at those places where the specific DNA targets on the specific chromosomal regions are located (ISH-spots). Sections were counterstained with (eosin and) hematoxylin.

***Evaluation***

Based on criteria, established in our laboratory at an earlier stage, sections were evaluated as follows (14). The sections of each case were checked to ensure that they were representative of the lesion and for preservation of its general architecture. The optimal slide to evaluate semi-quantitatively was considered to be the one with well demarcated (by hematoxylin) melanocytic nuclei (preserved morphology) in combination with the visualization of all targeted chromosome-regions present (clearly visible, discrete, well-separated ISH-spots). All lesional nuclei were first screened for the presence of more than 2 (>2) ISH-spots: two observers (PdW, HK) scored the lesions for the presence of nuclei with >2 ISH-spots, without counting, into the categories: a) aneuploidy suspected (+), b) equivocal (±), c) aneuploidy not suspected (−). In addition, areas were selected, which at screening appeared to have the most nuclei with >2 ISH-spots. Otherwise, random high power fields (HPF= x400) were selected. A minimum of 10 HPFs per lesion were examined and the number of nuclei with >2 ISH-spots per HPF was counted. Non-parametric statistics were applied.

## RESULTS

### *Unequivocal cases*

Table IA lists the data of the DNA-ISH experiments on the 15 histologically typical Spitz naevi. On screening, all lesions were scored as "aneuploidy not suspected" by both observers. All lesions, except for one (No. 4), had 2 or fewer nuclei with >2 spots as a maximum observed in one HPF. Case 4 showed 4 nuclei with >2 spots in one field (column D). These were nuclei of Spitz's multinucleated giant cells, some of which showed >3 spots. Not taking the giant cells in this case into account, the mean number of nuclei with >2 spots per HPF (10 HPFs counted) varied from 0 to 0.9 (mean 0.41, SD 0.26). A representative example is shown in Figure 1A.

Table IB lists the data of the DNA-ISH experiments on the 15 histologically typical nodular melanomas. On screening the lesions, nine were considered clearly "aneuploidy suspected", three were scored "not suspected" and three others were "equivocal". These results were significantly different from the results of Spitz naevi (Fisher's exact test, $p<0.001$). There was a correlation between the mean number of nuclei with >2 spots per HPF and the global assessment at screening (Spearman correlation coefficient: 0.87; $p= 0.0001$). The "not suspected" lesions showed a low mean number of nuclei with >2 ISH-spots. The mean number of nuclei with >2 spots per HPF (10 HPFs counted) ranged from 0.6 to 11.1 (mean 4.01; SD 3.4). This was different from the results in Spitz naevi (Mann-Whitney test, $p= 0.0001$). The data for the melanomas also differed from those of the Spitz naevi with regard to the maximal number of nuclei with >2 spots seen in one HPF (columns D; Mann-Whitney test, $p= 0.0001$)) and to the presence of nuclei with more than 3 spots (columns C; Fisher's exact test, $p= 0.02$). Also, the mean number of nuclei with >2 spots per HPF (column B) increased with the Breslow-thickness of the lesion (Spearmann correlation coefficient: 0.53, $p = 0.04$). A representative example is shown in Figure 1B.

### *Problematic cases*

Table II gives the data of the DNA-ISH experiments in the five cases of melanoma diagnosed in first instance as Spitz naevus. The mean number of nuclei with >2 spots per HPF ranged from 0.4 to 5.5. No nuclei with more than 3 spots were observed. A representative example is shown in Figure 1C.

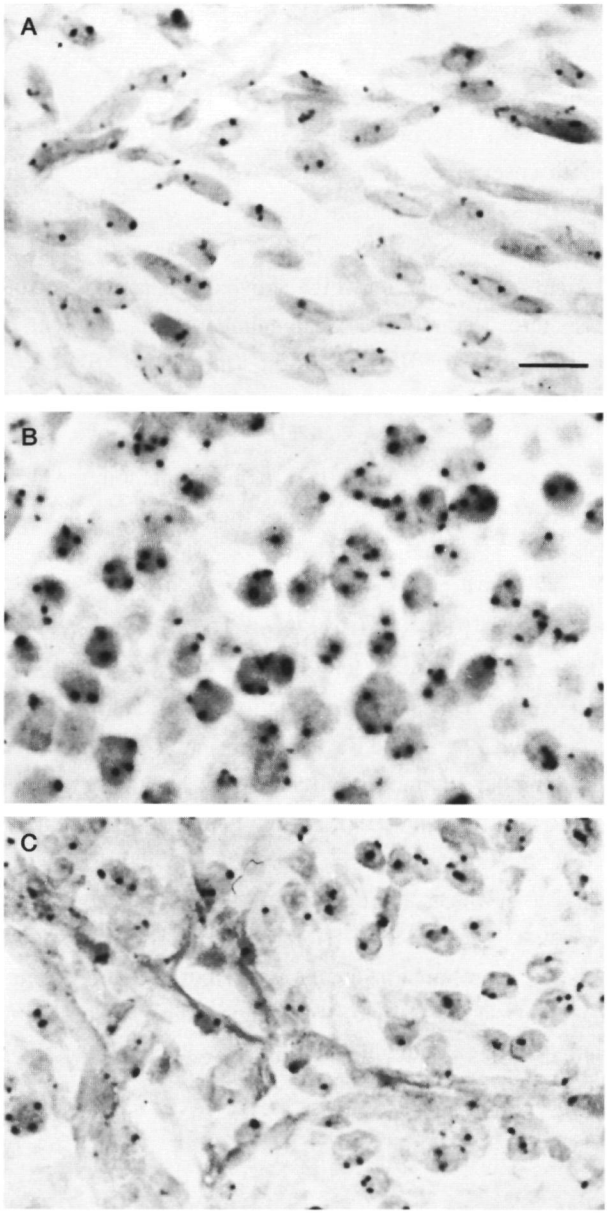


**Figure 1: DNA-ISH on melanocytic lesions.**

DNA-ISH on a typical Spitz naevus **(A)**, on a typical nodular melanoma **(B)** and on a melanoma initially misdiagnosed as Spitz naevus **(C)**. In B and C, nuclei with > 2 ISH-spots per nucleus are present, but not in A. For evaluation criteria see text. Bar = 20 micrometer.

A summary of the results in the different lesions on the mean number of nuclei with >2 spots per HPF is presented in Table III.

**Table III: Number of aberrant nuclei per HPF in melanocytic lesions studied (summary)**

| Number of nuclei with >2 ISH spots (mean per HPF) | Typical Spitz naevus* | Typical melanoma | Misdiagnosed melanoma |
|---|---|---|---|
| > 10 | | ● | |
| 5 - 10 | | ●●●●● | ●● |
| 2 - 5 | | ●●● | ● |
| 1 - 2 | | ●●●● | ● |
| 0.5 - 1 | ●●●●●● | ●● | |
| 0 - 0.5 | ●●●●●●●●● | | ● |

* Each circle represents one lesion

## DISCUSSION

The differential diagnosis between malignant melanoma and Spitz naevus is a well-known problem in dermatopathology (1). This problem relates especially to the vertical growth phase of primary melanoma. In superficial spreading melanoma with a nodular component, the vertical growth phase, a histological diagnosis of melanoma can often be made based on the characteristic features of the associated horizontal growth phase. On the other hand, in the case of a nodular melanoma, the diagnosis has to be made on the vertical growth phase alone. In view of these considerations, our study concentrated on the differential diagnosis of Spitz naevus and nodular melanoma. A definite diagnosis of malignant melanoma should be soundly based because of clinical implications that include: a) re-excision with possible mutilating effects, b) staging procedures, c) the follow-up regimen and d) examination of relatives in case of familial melanoma. In routine practice, consultation of more experienced (dermato-)pathologists may solve a number of diagnostic problems. However, even expert opinions on hematoxylin and eosin stained sections may differ. Several approaches have been put forward in such problematic cases in order to establish their malignant

nature. Rode et al, using immunohistochemical methods recently described a significant difference in mean staining intensity for S100 and NSE, comparing a group of Spitz naevi with a group of melanomas (11).

Most reports on the differential diagnosis between Spitz naevus and melanoma have focused on differences in DNA content (8-12). These have included image-cytometry on disaggregated nuclei or on nuclei in tissue sections, and flow-cytometry. The results of these studies indicate that Spitz naevi are euploid. In contrast, a considerable number of nodular melanomas is aneuploid, which is in concordance with data from cytogenetic studies (15-16). To the best of our knowledge, no karyotyping studies for Spitz naevi have been reported. This reflects the fact that it is impracticable to sample and culture or store fresh (frozen) tissue from every pigmented skin lesion in a routine setting. Additional diagnostic techniques should therefore be applicable on formalin-fixed, paraffin-embedded material.

Here we report on the positive identification of three out of five malignant melanomas that had previously been diagnosed as Spitz naevus. This was possible using microscopical visualization of aneuploidy by applying DNA-ISH on routinely processed lesions. The technique has several advantages. It requires no specific expensive equipment. It takes relatively little tissue and can be applied to small lesions. Each nucleus can be analyzed separately in its histological context because the architecture is preserved. This enables the reliable evaluation of known cell types. Since the marker of aneuploidy is visualized as the presence of three or more discrete spots within a counterstained nucleus, comparison with a normal diploid population is not needed. These factors may be regarded as advantages compared with the flow-cytometric and/or image-cytometric analysis of aneuploidy.

To support our data regarding ploidy assessed as by DNA-ISH, the DNA content of four Spitz naevi (Table IA: Nos 2, 3, 8 and 9) and 4 nodular melanomas (Table IB: Nos 9, 11, 12, and 15) was also assessed using image-cytometric analysis on Feulgen-stained tissue sections (data not shown). The ploidy of these lesions determined by this technique correlated well with the ploidy assessed by DNA-ISH: Spitz naevi showed DNA indices of 1.1 (No. 2), 0.93 (No. 3), 0.95 (No. 8) and 0.92 (No. 9) and nodular melanomas of 1.0 (No. 9; this case was not suspected for aneuploidy at DNA-ISH), 1.6 (No. 11), 1.7 (No. 12) and 2.2 (No. 15).

It is remarkable that the detection of supernumerary chromosomal aberrations based on only one target, on chromosome 1, already yields diagnostically

relevant information. Conceivably, the extension of the number of targets studied may improve the efficacy of the DNA-ISH approach. Future investigations should explore the appropriate choice and number of such targets. The rationale for chosing the 1q12 target on chromosome 1 is that this region is very often over-represented in melanoma, especially in advanced lesions (15-16). As a consequence those lesions which are aneuploid have mainly 3 or more of these targets per nucleus. A further practical reason is that the 1q12 target is quite large and can be visualized better than other targets in paraffin sections.

The nodular melanomas studied frequently showed aberrant nuclei and nuclei with >3 spots were also seen. However, occasional melanomas had counting results overlapping with those of the Spitz naevi. This was not unexpected, as a proportion of the melanomas have been reported to be euploid/diploid (7). Thus, the technique used here does not therefore provide an absolute distinction between melanomas and Spitz naevi, but can contribute to the identification of a considerable proportion of melanomas. Assessing and counting the number of aberrant nuclei in ten HPF provides indirect information on the ploidy of the lesion and thus on its nature. Using an arbitrarely chosen lower margin of at least three aberrant nuclei per HPF as a mean, out of five originally misdiagnosed cases, three would have been correctly designated as melanoma. This result on difficult lesions is quite revealing, as these cases represent major diagnostic problems in daily practice. With regard to the Spitz naevi, there are no reports which provide karyotyping data on these lesions. Flow- and image-cytometric studies show that they are diploid, with sometimes few hyperdiploid or polyploid cells (8-12). Our data suggest that in Spitz naevi, cytogenetic aberrations may be found, although in a very low percentage of cells. One case out of 15 showed a pattern which indicates a few polyploid cells within a diploid population. Chi et al also found such a pattern in 2 out of 20 Spitz naevi using a cytofluorometric analysis (10).

In conclusion, we have described here a new approach which helps positively to identify melanoma cases that are problematic on conventional light microscopy. As it is applicable on routinely processed lesions and does not require expensive equipment, it may be of practical use in the differential diagnosis between melanoma and Spitz naevus.

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# CHAPTER 7

# LOSS OF HETEROZYGOSITY DETECTED BY MICROSATELLITE ALLELOTYPING IN THE DIFFERENTIAL DIAGNOSIS OF MELANOMA VERSUS SPITZ NAEVUS

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**ABSTRACT**

We describe a study in which routinely processed tissues were used as starting material to perform allelotyping experiments on Spitz naevi and malignant melanomas in order to investigate whether the phenomenon of loss of heterozygosity (LOH), as a marker for the presence of so-called early or later genetic events, may be used for diagnostic purposes. We used 5 microsatellite markers, D3S1293, D6S271, D9S171, D10S187 and D18S70 to detect LOH at the chromosomal regions 3p, 6q, 9p, 10q and 18q, respectively. The technical procedure using automated fluorescense- based DNA sizing equipment was optimised for this application. Criteria for the interpretation of the output graphics, based on evaluation by naked eye assisted by calculating allele imbalance ratios, were established. The overall number of tests evaluable with regard to the presence of LOH, was comparable for melanomas (29) and Spitz naevi (27). Not-interpretability (34 tests) was due to homozygosity of the markers (21), to preferential PCR of a significantly smaller allele (5) or to unknown factors, probably related to poor quality DNA (8). While 5 out of 9 melanomas showed LOH at at least one locus, no LOH was found in the 9 Spitz naevi studied. In melanomas with LOH, either the chromosome 9 marker or chromosome 10 marker was always involved. As most mentioned problems can be circumvented by the application of more markers, we conclude that our results provide evidence that the detection of LOH using fluorescence-based technology can be valuable in the differential diagnosis of melanoma versus Spitz naevus, which merits a more extended study.

## INTRODUCTION

Although dissemination in cutaneous malignant melanoma occurs already at a relatively small tumour volume, the prognosis is excellent when this tumor is detected and excised at an early stage. Due to physicians and the general population being keen on early diagnosis, pathologists are presented with many melanocytic lesions for diagnosis (1). In the histopathology of cutaneous melanocytic proliferations, the discrimination between malignant and benign lesions can be difficult. Regularly, clinically relevant diagnostic difficulties are encountered, including the differential diagnosis of melanoma versus Spitz naevus. As also pathologists who are experienced in this field have problems here, there is a need for additional techniques to support the histopathological diagnosis and to minimise the number of so-called "impossible cases" (2). Such techniques should preferably be applicable on routinely fixed and embedded tissues.

Besides immunohistochemical and morphometrical studies, most reports dealing with the differential diagnosis of Spitz naevus versus melanoma are based on differences at the DNA level. In concordance with data from the cytogenetic literature, thick melanomas are often aneuploid as determined by flow- or image-cytometrical techniques, whereas Spitz naevi are reported to be euploid (3-6). This difference can be useful for diagnostic purposes and it formed for us the basis for a DNA-in situ hybridization study, aiming at the visualisation of numerical aberrations of the centromere region of chromosome 1 as an indicator of aneuploidy (7). However, already at the light microscopical level using haematoxylin & eosin (H&E) stained slides, the most difficult clinically relevant cases to diagnose as melanoma are those thick and intermediate-thickness lesions with a uniform nuclear appearance, i.e. melanomas with relatively small, non-hyperchromatic nuclei, mimicking benign naevi. These melanomas probably form a subgroup which are diploid or which have DNA-content-aberrations not measurable with current cytometric techniques. In such melanomas it may be assumed that, although rather gross numerical aberrations have not (yet) occurred, early genetic changes are present. So, the detection of these early events in melanomas (both thin and thick) may serve diagnostic purposes if these events are not found in Spitz naevi.

It has been reported for melanoma that deletions of chromosomal regions on chromosomes 9p and 10q, probably harbouring tumour suppressor genes, are among these early events (5, 8-11). On the other hand, detection at the nucleotide level of later events may, if already present, be of diagnostic value if not

occurring in Spitz naevi. Here, amongst others, deletions on chromosomes 3p, 6q and 11q have been reported (12-16). The detection of deletions by the study of loss of heterozygosity (LOH) has become wider applicable with the introduction of microsatellite markers. Microsatellites are small non-coding repetitive DNA sequences, highly variable in size, mostly highly polymorphic and widely present in the human genome. Using specific primers these sequences can be amplified by polymerase chain reaction (PCR) and the applicability to formalin-fixed, paraffin-embedded tissues offers possibilities for the study of archived materials (17).

In this study we explored whether LOH might serve diagnostic purposes in discriminating melanomas from Spitz naevi. We selected microsatellites based on data derived from karyotyping and molecular studies on melanomas (5-16). For Spitz naevi no karyotypes are described, while one LOH study was reported in the course of this study (18). We explored technical possibilities and problems with regard to the use of automated fluorescence-based DNA sizing equipment including the output interpretation with regard to the presence or absence of LOH (19). Using 5 microsatellite markers we found LOH in melanomas but not in Spitz naevi.

## MATERIALS AND METHODS

### *Melanomas and Spitz naevi: tissue selection*

For this LOH study we selected nine cases of histopathological unequivocal, mostly thick nodular melanomas, not derived from patients with the dysplastic naevus syndrome. These cases were selected because the chance of finding DNA aberrations here was considered high, which was regarded important with respect to the technical aspects to be evaluated. Also it was postulated that by studying the selected melanomas, the outcome with regard to the diagnostic efficacy would indicate the merit of a more extented study. For comparison, nine cases of Spitz naevi were selected, three of which showed an atypical nuclear appearance. Characteristics of the lesions selected are summarized in Table I. All lesions were from different individuals and taken from the archives of the routine pathology practice, implying that all tissues were fixed in buffered formalin (4%) with fixation times varying from about 16 to 64 hours. Melanocytic lesions were selected which were at least moderately cellular and which had a not too brisk lymphocytic infiltrate, in order to reduce the chance of substantial contamination of tumour DNA with normal DNA. Contamination by non-tumour cells was

estimated microscopically: the selected tumour tissues contained at least 60 percent tumour cells. Melanomas and Spitz naevi were microdissected without paying attention to the presence of areas with a different morphological appearance, thus incorporating the possibility of mixing different populations from a heterogeneous lesion. Although morphological heterogeneity was present in melanomas, Spitz naevi had a homogeneous cytonuclear appearance. Depending on the size of the tissue of interest, 4-50 consecutive sections of 10 $\mu$m thickness were used. For control non-tumourous tissue, normal skin from the same surgical excision was used, either (if present) from the paraplast blocs with normal skin (negative resection margins) or microdissected, guided by a parallel H&E-stained slide, from the same bloc in which the tumour was present (non-tumourous lateral margins). For one case (no. 1) a tumour-negative lymph node was used as a control. In case of the tumours, the non-tumourous (basal and/or lateral) dermal and epidermal parts were removed by microdissection after mounting the sections on glass slides. In addition, in relatively thin Spitz naevi, the supra-tumoural epidermis was scraped away, in order to avoid substantial contamination with keratinocytes. After selection by microdissection, the tissue parts of interest were put into a 1.5 ml Eppendorf tube.

*DNA isolation*

The DNA extraction procedure was performed using the Puregene DNA isolation kit (Gentrasystems, Triangle Park, USA). The selected tissues were treated for 18 hours at 55°C with 0.5 mg/ml proteinase K in 500 $\mu$l cell lysis solution according to the recommendations of the manufacturer. Further digestion was performed for 48 hours at 37°C. After cooling-down the samples to room temperature, 150 $\mu$l of protein precipitation solution was added, followed by mixing for 20 seconds and centrifugation (14,000xg, 4°C). To the supernatant, 500 $\mu$l isopropanol (100%) was added followed by gentle mixing and centrifugation (14,000xg, 4°C) for DNA precipitation. After washing the pellet with 500 $\mu$l ethanol (70%) at 4°C and centrifugation (14,000xg, 4°C), DNA was air-dried. Fifty $\mu$l of DNA hydration solution was added for DNA solubilisation for 60 minutes at 65°C. DNA was stored at 4°C. Since in starting experiments with deeply pigmented tumours we obtained sufficient PCR products and also because the lesions selected for this study were not very heavily pigmented, we did not include a melanin extraction step.

*PCR-analysis*

Amplification of the targets was performed using standard procedures and primers labelled with fluorescent phosphor-amidites, linked to the forward primer (commercially obtained from Perkin-Elmer Corporation, Warrington, UK). Markers used are listed in Table II together with data on genomic localisation, length and degree of heterozygosity. Samples were denatured for 5 minutes at 93°C and passed through 35 or sometimes up to 40 cycles of amplification consisting of 45 seconds denaturation at 92°C, 45 seconds primer annealing at 50°C, 60 seconds elongation at 72°C and a final extension of 5 minutes at 72°C (Mastercycler, Eppendorf, Germany). After PCR and evaluation of the products on a agarose gel, samples were denatured with formamide and subjected to electrophoreses on a 6% polyacrylamide gel in a 373 DNA Sequencing System (Applied Biosystems, Perkin-Elmer Corporation, Foster City, USA). In this system the fluorescence-labeled PCR products were size-separated and detected after excitation of the labels. The emitted fluorescence, after filtering, was detected by a photomultiplyer system and the banding pattern was analyzed by computer software (Genescan, Applied Biosystems, Perkin-Elmer Corporation). This multicolour system offers the possibility for running a size standard in the sample lane and combines possibilities for quantification of the amplified product with a rather large range of response linearity. As an output, electrophoresis bands were plotted graphically with fluorescence intensity plotted per basepair number. Values were retrieved on the basepair number at the peak position, on fluorescence level of the peak top and area (surface) of the peak, allowing a quantitative comparison of tumour and non-tumour-derived data.

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**Legend to Table I (next page)**

[a]: SSM = Superficial Spreading Melanoma; NM = Nodular Melanoma; SNm = Spitz Naevus, mixed spindle and epithelioid; SNe = Spitz Naevus, epithelioid cell predominant; SNs = Spitz Naevus spindle cell predominant.

[b]: In millimeters according to Breslow; for Spitz naevi measured similarly as for melanomas.

[c]: H: homozogous; +: heterozogous with LOH; -: heterozygous and no LOH; * and #: no evaluation possible (see Results).

[d]: Fraction of informative markers with LOH in the given case.

[e]: Not classical appearance: atypical Spitz naevi.

**Table I: Loss of heterozygosity in melanomas but not in Spitz naevi**

| no | type of lesion[a] | thick-ness[b] | LOH[c] | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | D3 S1293 | D6 S271 | D9 S171 | D10 S187 | D18 S70 | LOH[d] |
| 1 | SSM | 7.0 | - | # | H | + | - | 1/3 |
| 2 | NM | 4.2 | - | - | H | - | H | 0/3 |
| 3 | NM | 4.5 | - | - | + | H | - | 1/4 |
| 4 | NM | 7.0 | - | + | H | + | - | 2/4 |
| 5 | NM | 7.5 | H | H | H | - | - | 0/2 |
| 6 | NM | 4.2 | * | H | - | + | + | 2/3 |
| 7 | NM | 4.5 | - | - | * | - | - | 0/4 |
| 8 | NM | 5.5 | + | - | + | H | - | 2/4 |
| 9 | NM | 2.15 | - | - | H | H | * | 0/2 |
| | LOH per marker | | 14% | 17% | 66% | 50% | 14% | |
| 10 | SNm | 1.1 | - | - | - | H | - | 0/4 |
| 11 | SNm | 2.6 | - | - | - | - | - | 0/5 |
| 12 | SNe[e] | 1.9 | # | # | # | # | # | 0/0 |
| 13 | SNe | 1.4 | - | - | H | - | - | 0/4 |
| 14 | SNs | 3.0 | * | - | H | - | - | 0/3 |
| 15 | SNm[e] | 1.9 | - | H | - | H | H | 0/2 |
| 16 | SNe | 1.2 | - | - | - | # | # | 0/3 |
| 17 | SNm | 1.1 | - | H | * | - | - | 0/3 |
| 18 | SNe[e] | 1.5 | - | - | - | H | H | 0/3 |
| | LOH per marker | | 0% | 0% | 0% | 0% | 0% | |

*Interpretation*

Interpretation of the results was based on evaluation by naked eye of plotted graphics assisted by calculating the allelic imbalance ratio between tumour and control tissue, using data on peak top level and peak area.

## RESULTS

The main results of the described experiments are summarized in Table I. In figure I plotted graphics are presented for three cases. From all cases DNA could be obtained from both the tumour and the control tissue, even if the starting material was little. There was a correlation between the estimated amount of microdissected tumour cells and the amount of DNA isolated from the tissues. PCR- and electrophoresis conditions were adjusted in such a way that optimal results were obtained. Per marker (primer set) optimal conditions were established by varying the $MgCl_2$ concentration in the PCR. For some samples of large lesions the isolated DNA had to be diluted before PCR to obtain optimal results. On the other hand, for small amounts of dissected tissue, up to 40 cycles had to be used to obtain sufficient product for electrophoresis. The amount of tumour DNA and control DNA of a pair was adjusted to similar levels as much as possible. The extra extension step at 72°C allowed for a better interpretation of the final graphic output. Per case and marker, a repeated experiment under optimal conditions was performed with reproducible results. Whereas most data were evaluable, some results were obtained which did not allow a conclusive interpretation. Five types of results were formulated: 1) homozygous, 2) heterozygous with LOH, 3) heterozygous and no LOH, 4) data not interpretable with regard to LOH due to a large difference in length between the two alleles with highly preferential PCR of the smaller allele, 5) data not interpretable with regard to (loss of) heterozygosity for unknown factors, probably representing poor quality DNA in combination with PCR-procedure derived factors, resulting in non-typical, non-explainable peak patterns.

With regard to the informativeness of the different markers in the tumours (both melanomas and Spitz naevi) the percentage of heterozygosity in evaluable cases was 93% for D3S1293; 75% for D6S271; 53% for D9S171; 63% for D10S187 and 80% for D18S70, which was somewhat higher than reported for the chromosome 3 marker but considerably lower for the chromome 9 and 10 markers (see Table II). This discrepency is probably due to the limited number of

lesions evaluated in this study.

In melanomas 8 out of 29 informative markers showed LOH (28%). LOH for marker D3S1293 was found in 1 out of 7 cases (14%). For the markers D6S271, D9S171, D10S187 and D18S70 these percentages were 17, 66, 50 and 14 respectively. Five out of 9 melanomas showed LOH at at least one locus (56%). There was no correlation between the estimated degree of contamination of tumour cells with non-tumour cells and the absence of LOH.

In the group of Spitz naevi no LOH was found: none of eight evaluable cases showed allelic loss on informative loci, including those with nuclear atypia. The total number (27) and the distribution of the informative markers in Spitz naevi and also the degree of contamination with non-tumour cells was comparable to that found for melanomas.

**Table II: Genomic localisation, heterozygosity and length of microsatellite markers used***

| | D3S1293 | D6S271 | D9S171 | D10S187 | D18S70 |
|---|---|---|---|---|---|
| chromosomal region | 3p24.2-ter | 6q25.2 | 9p21 | 10q22-ter | 18q23 |
| heterozygosity (%) | 80 | 85 | 80 | 84 | 83 |
| allele length (bp) | 116-144 | 166-208 | 159-177 | 92-114 | 111-126 |

* based on the genome database: http://gdbwww.gdb.org/

## DISCUSSION

In this paper we report on the use of the detection of LOH in diagnostic melanocytic pathology. For this purpose microsatellite allelotyping using non-radioactive, fluorescence-based automated DNA-sizing technology was applied. The knowledge on genetic mechanisms related to the initiation and progression of human malignancies has increased substantially with studies on the selective loss of DNA sequences in tumours in comparison with normal tissues. Loss of genetic material in tumours has been taken as evidence for the involvement of tumour suppressor genes. The analysis of highly polymorphic microsatellites by PCR

facilitated LOH-studies and the small size of these markers was found to be an advantage in the studies of archival tissues, since they proved relatively insensitive to autolysis and to routine procedures used in pathology, including formalin fixation and paraffine embedding. These characteristics imply that microsatellite analysis might also be of interest for diagnostic pathology, moreover because only small samples are needed. We investigated whether the detection of LOH may serve diagnostic purposes in the differential diagnosis of melanoma versus Spitz naevus. This study was designed to explore this hypothesis in a group of lesions that in theory would harbour the highest chance of positive results, this in order to assess technical aspects next to the diagnostic issue.

In the course of this study we learned what difficulties are related to the techniques used when applied to formalin-fixed, paraffin-embedded tissue-derived DNA. The inherent difficulty of discriminating between presence or absence of LOH was noted. This is probably based on problems related to contamination of tumour with non-tumourous tissue due to the inevitable presence of normal cells in tumour samples. To this end, several factors were taken into consideration at the start of this study. Tumour lesions were microdissected guided by parallel H&E-stained slides and the relative extent of contamination of the tumour parts with lymphocytes and stromal components was estimated. Cases were selected in the first place for the absence of a brisk infiltration of lymphocytes or an extensive stromal component. Based on titration experiments using autoradio-graphical methods, Gruis et al. described that for a tumour with LOH, a safe margin to handle to avoid false negative cases is to require a minimum of 40% of tumour cells in the sample (20). However, as we do not know the quality of the tumour DNA compared to the control DNA we feel uncertain to formulate that contamination was only a minor problem in our experiments, although lesions were selected for an estimated percentage of tumour cells of 60 or more.

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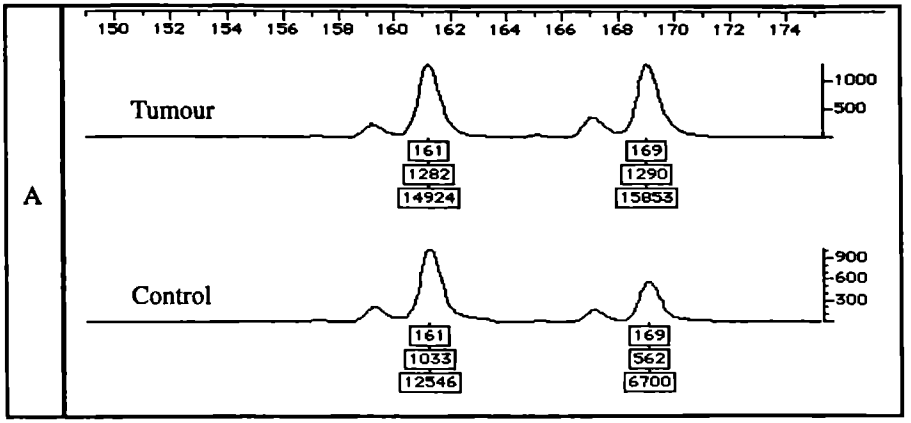
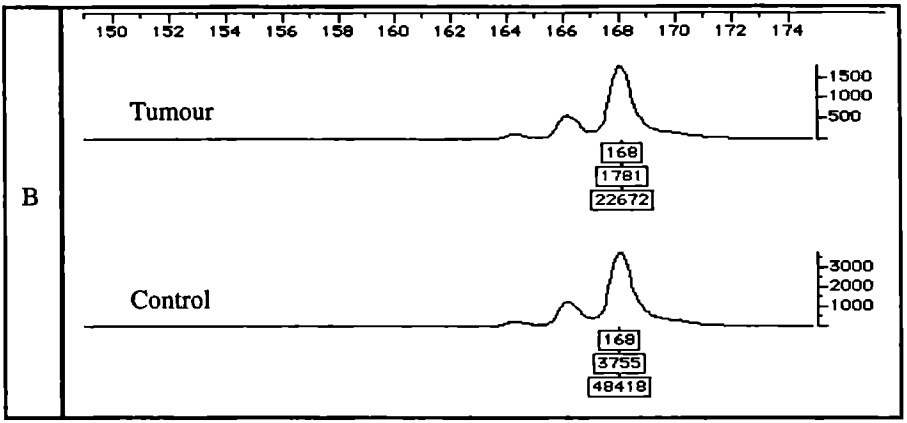
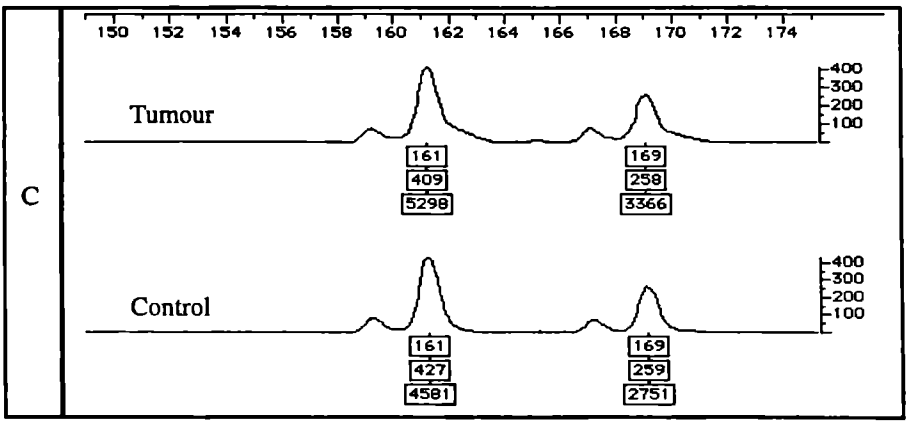
**Figure 1 (next page): LOH presence or absence in melanocytic lesions.**
Examples of plotted curves representing electrophoresis patterns, showing fluorescence intensity (Y-axis) versus basepair-number (X-axis). The number in the upper rectangle represents the basepair number position of the peak, the middle rectangle shows the peak top level and the lower rectangle indicates the area of the peak. **A:** malignant melanoma with LOH for marker D9S171 (in the tumour, the 161 bp peak is too small compared with the ratio between the peaks in the control); **B:** melanoma homozygous for marker D9S171; **C:** Spitz naevus without LOH for marker D9S171.


A
150 152 154 156 158 160 162 164 166 168 170 172 174
Tumour
1000
500
161
1282
14924
169
1290
15853
Control
900
600
300
161
1033
12546
169
562
6700


B
150 152 154 156 158 160 162 164 166 168 170 172 174
Tumour
1500
1000
500
168
1781
22672
Control
3000
2000
1000
168
3755
48418


C
150 152 154 156 158 160 162 164 166 168 170 172 174
Tumour
400
300
200
100
161
409
5298
169
258
3366
Control
400
300
200
100
161
427
4581
169
259
2751

In addition, heterogeneity in lesions may be a factor diluting the targeted aberrant DNA. In diagnostic practice, these considerations would imply that the absence of LOH should always be regarded with care in cases with an estimated small percentage of tumour cells. One approach may be a precise quantification of contaminating cells in parallel slides. Further, to obtain insight into problems related to contamination and heterogeneity, selection procedures using laser microdissection equipment may be valuable. In line with this, our results imply promising perspectives with regard to the use of microsatellites in studies on melanocytic tumour progression, if combined with laser dissection. This because in situ techniques to study small deletions in routinely processed tissues are not available (21,22).

Another technical aspect of importance is the PCR procedure. It remains difficult to perform reliable quantitative PCR experiments in which the relative amount of product of either allele reflects the relative amount of starting material of these alleles. To reduce these problems, we always compared PCR products of tumour and control DNA pairs that were derived from the same experiment, using the same number of cycles and that contained a similar amount of PCR product, as judged on agarose gels. Moreover, only data that were reproducible in independent experiments were used for analysis. The preferential amplification of the smaller allele in cases with a large difference in basepair length of the alleles prohibited a conclusive analysis of the possible loss of the larger allele (cases marked with an asterix in Table I). Although a minimal difference in length (one dinucleotide) would thus be preferable, in the PCR always a product of one dinucleotide smaller than the genuine allele is formed. This results in overlapping curves for the alleles in such heterozygous cases, complicating the evaluation (part of the cases marked # in Table I). Cases with intermediate differences in length of the alleles proved to be best suitable to arrive at a conclusive interpretation.

As expected also some unexplainable and thus uninterpretable results were encountered, including all markers tested in case no 12. These results were probably due to poor quality of the DNA caused by autolytic degradation or formalin fixation (17). Also PCR artefacts may be important here, but, although in general more PCR artefacts are introduced when starting with a smaller amount of DNA, three cases in our series with the smallest amount of starting tissue showed proper curves. Notwithstanding the mentioned problems, it was

possible to classify most cases with regard to (loss of) heterozygosity.

While five out of nine melanomas showed LOH for at least one of five markers used, no LOH was found in Spitz naevi. Although we studied only a limited number of melanomas, our results are in line with data from the literature, especially with regard to the high percentage of LOH found for the 9p and 10q markers, underlining the frequent involvement of these regions in melanoma (6). Unfortunately, in our study the markers used in these regions (D9S171 and D10S187) were homozygous in a rather large proportion of melanomas. We assume that if more melanoma cases would have been heterozygous for the chromosome 9 marker, more melanoma cases with LOH would have been found. So, in diagnostic terms, the sensitivity of the detection of LOH to support the diagnosis of melanoma is complicated by homozygosity of the markers. The use of more markers per chromosomal region of diagnostic interest may circumvent this problem. Also, more results on one relevant region may corroborate each other. Like the use of more markers per region, the use of markers on more chromosomal regions may improve the sensitivity with regard to the detection of LOH. Although in our set it appeared that the study of more regions besides 9p and 10q provided no additional information, in the literature melanoma cases are described with LOH detected only in regions other than 9p or 10q (6,16). As at diagnostic evaluation of a lesion one does not know in which specific stage of genomic abnormality a presumed melanoma has arrived, both early markers and late markers may harbour diagnostic information. Further studies may clarify the additional value of including more early and/or late markers.

In our group of Spitz naevi we did not find LOH, which would imply a high specificity for melanoma of the finding of LOH. In the course of this study, Healey et al reported on LOH in 2 out of 27 Spitz naevi, both on chromosome 9p (one out of 20 informative cases with marker D9S171 and one out of 14 with marker D9S162). No further LOH in Spitz naevi was found by these authors, using 12 markers targeting those 7 chromosomal regions (3p, 6q, 9p, 9q, 10q, 17p and 18q) which in their group of melanomas showed the highest percentage of LOH (18). With regard to this it is interesting that our two evaluable atypical Spitz Naevi did not show LOH. Nevertheless, as deletions on 9p also otherwise seem to be not truly malignancy specific as they were also, although rarely found in dysplastic naevi, LOH in regions other than 9p may be less sensitive but more specific for melanoma (18). So, both from a diagnostic and cell biological interest

a future study might, next to the incorporation of a larger number of unequivocal as well as misdiagnosed melanomas and Spitz naevi, also include dysplastic naevi, all with a well documented follow-up. With regard to patient care, besides the diagnostic benefit, another interesting perspective of LOH detection is the identification of additional prognostic factors for melanoma patients, both derived from the primary tumour as well as on the basis of the detection of free tumour DNA in blood (21,23).

We conclude on the basis of our results that the detection of LOH through microsatellite-allelotyping using fluorescence-based technology may be valuable in the differential diagnosis of melanoma versus Spitz naevus, which merits further study.

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# SUMMARY

The melanin pigment in the human skin is produced by melanocytes. Naevocellular naevi may be regarded as benign neoplasms of melanocytes. Malignant neoplasms of melanocytic cells are termed "malignant melanoma", synonymously used with "melanoma". Two pathways of tumourigenesis in melanoma have been proposed: a de novo proliferation of epidermal melanocytes can occur or a more gradual progression via melanocytic naevi as an intermediate stage. An intermediate lesion in melanocytic tumour progression between the common acquired naevus and melanoma has been recognized and termed "dysplastic naevus". This lesion is considered a clinically relevant potential precursor of melanoma. Although melanoma is not a "big killer", in young adults it is an important cause of death and for various reasons, many clinical and basic research activities have focussed on this type of cancer. From a clinical point of view, immunotherapy using transduced human cells, sentinel node procedures and refined isolated limb perfusion protocols are examples of innovative patient care. With respect to tumour biological research, malignant melanoma and its precursors form an attractive model because of the possibility to study molecular aspects of tumour progression using a series of discrete stages, which can be identified histologically by the pathologist. For the general pathologist melanocytic lesions form a substantial part of the daily work, which may be related to an increased interest in recognizing melanoma at a curable stage and in recognizing precursor stages. Relevant clinico-pathological characteristics of melanoma, the dysplastic naevus and another type of naevus i.e. the Spitz naevus, are described in the general introduction (chapter 1), since they create a substantial part of the problems encountered by diagnostic pathologists.

In chapter 2, the character and relative extent of difficult areas in melanocytic pathology is described as deducted from the cases submitted to the Pathology Panel of the Dutch Melanoma Working Group. A consecutive series of 1069 referral cases, submitted in 1992 through 1994 were analysed. Of the 158 lesions classified as invasive melanoma by the referring pathologists, 22 were considered to be benign by the panel. Conversely, 108 invasive melanomas (panel diagnosis) had originally been considered as benign naevus or melanoma in situ. Most diagnostic difficulties were encountered with dysplastic naevi and Spitz naevi and the results give guidance to the focussing on efforts to reduce the specific problems regarding these lesions. To this end, in this thesis histomorphological,

as well as immunohistochemical, DNA-in situ hybridization (DNA-ISH) and allelotyping approaches are described.

Chapter 3 describes the attempt made, through an interobserver study among experts in the field of melanocytic pathology in Europe, to come up with a set of best reproducible histomorphological parameters in melanocytic lesions leading to the highest chance of obtaining a reproducible, although still arbitrary histological "boundary" between dysplastic naevi and common acquired naevi. This is important because the histological identification of a dysplastic naevus may indicate that, like melanoma itself, the patient has an increased risk of developing a (subsequent) melanoma, possibly in the context of the dysplastic naevus syndrome, the latter potentially also of importance for the family of the patient. Although there is still discussion on the histological criteria which are obligatory for the diagnosis of a dysplastic naevus, basically, the selection and weight of a feature for a diagnostic purpose rests on its validity in terms of the discriminatory value (efficacy) and the reproducible recognition among observers. To investigate this, ten (dermato)pathologists studied fifty cutaneous melanocytic lesions including common naevocellular naevi, dysplastic naevi, melanomas in situ and invasive primary melanomas. Using a standardized form, 20 defined histopathological features were scored (semi)quantitatively. Concordance of diagnosis, efficacy and reproducibility of features were investigated. Dysplastic naevi were distinguished well from the other entities (mean $P_o$ 0.87). Agreement on the degree of atypia of dysplastic naevi was low. The reproducibility of the scoring was best for the following features: irregular nests, lymphohistiocytic infiltrate, marked junctional proliferation and large nuclei. The overall values of these features to discriminate between dysplastic naevi and non-dysplastic naevi were better than for the other features studied. Using the presence of at least 3 of the 4 features as a condition for the diagnosis of dysplastic naevi, values for sensitivity, specificity, positive and negative predictive values were 0.86, 0.91, 0.96 and 0.73, respectively. On the basis of the results these features seemed best suited as histological criteria for the diagnosis of dysplastic naevi.

The dysplastic naevus as a simulant of melanoma is another histopathological problem. Especially the differentiation between the deeper dermal part of a malignant melanoma versus the naevus remnant underlying a micro invasive, radial growth phase melanoma is a clinically relevant problem as it has major impact on the Breslow thickness and thus on prognosis and clinical management.

As a basis towards a possible application in this differential diagnosis, chapter 4 presents a study on the expression of the epidermal growth factor receptor (EGFR) on melanocytic cell lines and tissues at the protein and mRNA level, in order to determine the use of EGFR as a possible progression marker. EGFR expression in six human melanoma cell lines was compared by utilizing the monoclonal antibodies 2E9, 425 and 225, applying four immunocytochemical staining procedures. A three-step immunoperoxidase method using the monoclonal antibody 2E9 proved the most sensitive. Staining intensities, estimated semi-quantitatively, correlated well with the quantitative data obtained by multiple point ligand binding assays which showed that EGFR expression in the highly metastasizing cell lines MV3 and BLM was at least 40 times higher than in the cell lines IF6, 530, M14 and Mel57 which do not or only sporadically metastasize after subcutaneous inoculation into nude mice. Expression on the mRNA level by Northern blot analysis was also in agreement with these results. Immunohistochemical staining of a large series of human cutaneous melanocytic lesions showed differential EGFR expression in various stages of melanocytic tumour progression: 19% of common naevocellular naevi, 61% of dysplastic naevi, 89% of primary cutaneous melanomas and 91% of melanoma metastases showed staining of melanocytic cells. It was concluded that EGFR expression increases in melanocytic tumour progression.

Chapters 5-7 concern approaches which can be used to reduce the number of cases with an unpredictable behaviour in the differential diagnosis of melanoma versus Spitz naevus. To this end, DNA-ISH and allelotyping experiments were performed using routinely processed, formalin-fixed, paraffin-embedded tissues.

In chapter 5 a study on the feasibility of DNA-ISH on melanocytic cell lines and tissues is described, in order to be able to study genomic changes and heterogeneity during melanocytic tumour progression. The possibility to detect numerical aberrations with respect to the (peri-)centromere regions of chromosomes 1 and 7 in intact nuclei of two human melanoma cell lines with different metastatic behavior in nude mice, was studied. In addition, paraffin sections from xenograft lesions were used, obtained by inoculation of these cell lines into nude mice (subcutaneous tumours and spontaneous lung metastases). Paraffin sections from the original primary cutaneous melanoma (with a subepidermal and a dermal part) and two loco-regional metastases were also studied, one of which was the source for the cell lines. With regard to the cell

lines, the ISH analysis correlated well with data obtained by ISH on metaphase spreads. Differences between the lines, which could not be detected by flow-cytometric or conventional karyotyping analysis, included data suggestive of a polyploid subpopulation and an extra copy of chromosome 7 in the metastasizing cell line. The polyploid population could be detected also in the paraffin sections of the corresponding subcutaneous xenografts and lung metastases in the mice. Both areas in the patients' primary melanoma could be evaluated separately and showed similar supernumerary aberrations of the chromosome-specific targets. These abnormalities matched those found in both metastases. The results demonstrated that DNA-ISH can be used to visualize genomic abnormalities at the single cell level in melanocytic nuclei in their natural context, which made it a possible tool in the histopathology of melanocytic lesions.

In chapter 6, a study is described on the possible implementation of DNA-ISH in the histological differential diagnosis between Spitz naevus and malignant melanoma. To this end routinely processed paraffin sections of 15 typical Spitz naevi, 15 typical nodular melanomas, and five cases originally misdiagnosed as Spitz naevus but which later metastasized and were reclassified as melanoma were analysed using DNA-ISH. Microscopical semi-quantitative evaluation revealed that the number of nuclei with supernumerary aberrations of the centromere region of chromosome 1, suggestive for aneuploidy, was significantly different in Spitz naevi and nodular melanomas. The mean number of aberrant nuclei per high power field was 0.41 and 4.01, respectively. On applying the results of the typical lesions to the equivocal, originally misdiagnosed lesions, three out of five could be identified as melanoma. These results suggested that the application of DNA-ISH may contribute to the positive identification of melanomas in the differential diagnosis with Spitz naevus.

In chapter 7 a study is described in which routinely processed tissues were used as starting material to perform allelotyping experiments on Spitz naevi and malignant melanomas in order to investigate whether the phenomenon of loss of heterozygosity (LOH), as a marker for the presence of so-called early or later genetic events, may be used for diagnostic purposes. Five microsatellite markers, D3S1293, D6S271, D9S171, D10S187 and D18S70 were used to detect LOH at chromosomal regions 3p, 6q, 9p, 10q and 18q, respectively. The technical procedure using automated fluorescense-based DNA sizing equipment was optimised for this application. Criteria for the interpretation of the output

graphics were established, based on evaluation by naked eye assisted by calculating allelic imbalance ratios. The overall number of tests evaluable with regard to the presence of LOH, was comparable for melanomas (29) and Spitz naevi (27). Not-interpretability was due to homozygosity of the markers (21) or to preferential PCR of a significantly smaller allele (5) or to factors not known, probably related to poor quality DNA (8). While 5 out of 9 melanomas showed LOH at at least one locus, LOH was not found in the 9 Spitz naevi studied. In melanomas with LOH, either the chromosome 9 marker or chromosome 10 marker was involved. As most mentioned problems can be circumvented by the application of more markers, it was concluded that the results indicate that the detection of LOH may be valuable in the differential diagnosis of melanoma versus Spitz naevus, meriting further study.

Summing up, in order to handle specific problems in melanocytic pathology, in this thesis molecular and morphological approaches are described. Some results of the studies are already implemented while others are of interest for future implementation in diagnostic melanocytic pathology.

# SAMENVATTING

Het melanine pigment in de menselijke huid wordt geproduceerd door melanocyten. Naevocellulaire naevi (moedervlekken) kunnen worden beschouwd als goedaardige nieuwvormingen van melanocyten. Kwaadaardige nieuwvormingen van melanocytaire cellen worden "maligne melanoom" genoemd of kortweg "melanoom". Voor de ontstaanswijze van melanomen worden twee routes voorgesteld: één direct vanuit epidermale melanocyten en een andere middels een stapsgewijze progressie via melanocytaire naevi. Bij melanocytaire tumorprogressie is een intermediaire afwijking tussen een naevus naevocellularis van het gebruikelijke type en een melanoom beschreven, die "dysplastische naevus" is genoemd. Deze laesie wordt beschouwd als een mogelijke voorloper van het melanoom. Ook wordt deze als een indicator voor een verhoogd risico op het ontstaan van een melanoom gebruikt bij het klinisch onderzoek. Ofschoon het melanoom niet een "big killer" is, is het bij jonge volwassenen een belangrijke oorzaak van overlijden en wordt er om verschillende redenen veel klinisch en basaal onderzoek verricht aan dit type kanker. Vanuit klinisch opzicht zijn immunotherapie middels vaccinatie met genetisch gemanipuleerde cellen, beperkte lymfklierdissectie (schildwacht-lymfklier-excisie) en geïsoleerde perfusie van de ledematen voorbeelden van innovatieve patiëntenzorg. Ook bij het tumorbiologisch onderzoek vormen het melanoom en de voorloper stadia een aantrekkelijk model vanwege de mogelijkheid tot het bestuderen van moleculaire aspecten in een reeks opeenvolgende stadia, die histologisch door de patholoog geïdentificeerd kunnen worden. Voor de algemeen patholoog vormen melanocytaire afwijkingen een substantieel deel van het dagelijkse werk, waarschijnlijk ook samenhangend met een toegenomen aandacht voor het herkennen van melanomen in een curatief behandelbaar stadium alsook voor het herkennen van voorloper stadia. Relevante klinisch-pathologische kenmerken van het melanoom, de dysplastische naevus en een ander type naevus, te weten de Spitz naevus, worden beschreven in de algemene inleiding (hoofdstuk 1) omdat deze afwijkingen een substantieel deel uitmaken van de problemen bij melanocytaire afwijkingen waarmee pathologen geconfronteerd worden.

In hoofdstuk 2 wordt de aard en de relatieve omvang beschreven van diagnostische problemen bij melanocytaire afwijkingen op basis van een reeks gevallen die werd toegezonden aan het pathologen-panel van de Nederlandse Melanoom Werkgroep. Een opvolgende reeks van 1069 gevallen uit 1992, 1993 en 1994

werd geanalyseerd. Van de 158 afwijkingen die door de inzendende pathologen geclassificeerd werden als melanoom, werden er 22 door het panel als goedaardig beschouwd. Omgekeerd bleken er 108 melanomen (panel diagnose) oorspronkelijk beschouwd te zijn als een naevus of als een melanoma in situ. De diagnostische problemen deden zich vaak voor bij dysplastische naevi en Spitz naevi. Op basis hiervan kan nascholing en aanvullend onderzoek gerichter gedaan worden. Het in dit proefschift uitgevoerde histomorfologische als ook immunohistochemische, DNA-in situ hybridisatie (DNA-ISH) en allelotyperingsonderzoek is tegen deze achtergrond verricht.

Hoofdstuk 3 beschrijft een interobserver studie bij Europese pathologen die ervaren zijn in het veld van de melanocytaire pathologie. Het doel was om de best reproduceerbare parameters, zoals gebruikt bij de diagnostiek van naevi, te onderkennen. Door gebruik te maken van deze parameters bestaat immers de hoogste kans op het verkrijgen van een reproduceerbare, alhoewel nog steeds arbitraire histologische grens tussen een dysplastische naevus en een naevus naevocellularis van het gebruikelijke type. Dit is belangrijk omdat de histologische diagnose "dysplastische naevus" net zoals de diagnose "melanoom" aangeeft dat de patiënt een verhoogd risico heeft op het ontwikkelen van een (volgend) melanoom, mogelijk in de context van een dysplastisch naevus syndroom. Dit laatste is mogelijk ook van belang voor de familie van de patiënt. Hoewel er nog steeds discussie gaande is over de histologische criteria die obligaat zijn voor de diagnose dysplastische naevus, is de waarde van een kenmerk voor een diagnostische toepassing gelegen in de bruikbaarheid, dat wil zeggen a) in het onderscheidend vermogen (efficacy) en b) in de reproduceerbare herkenning door verschillende waarnemers. Om dit te onderzoeken bestudeerden tien (dermato)-pathologen vijftig melanocytaire huidlaesies waaronder naevocellulaire naevi van het gebruikelijke type, dysplastische naevi, melanomas in situ en invasieve primaire melanomen. Gebruikmakend van een gestandaardiseerd formulier werden twintig goed omschreven histopathologische kenmerken (semi)kwantitatief gescoord. Onderzocht werden overeenkomst in diagnose alsmede efficacy en de reproduceerbaarheid van de kenmerken. Dysplastische naevi werden goed onderscheiden van de andere entiteiten (gemiddelde $P_o$ 0.87). De overeenstemming over de mate van atypie in dysplastische naevi was echter laag. De reproduceerbaarheid was het beste voor de volgende kenmerken: irregulaire nesten, lymfohistiocytair infiltraat, duidelijke grensvlak activiteit en grote kernen. Op basis van juist ook deze kenmerken bleken dysplastische en

niet-dysplastische naevi goed onderscheiden te kunnen worden. Indien de aanwezigheid van tenminste drie van de vier genoemde kenmerken gebruikt werd als een voorwaarde voor de diagnose dysplastische naevus bedroegen de gevoeligheid, specificiteit en positieve en negatieve voorspellende waarde respectievelijk 0.86, 0.91, 0.96 en 0.73. Op basis van deze resultaten werden deze kenmerken beschouwd als de meest geschikte bij het diagnostiseren van dysplastische naevi.

De dysplastische naevus kan door de aanwezigheid van cellulaire en architecturele atypie sterk op een melanoom lijken, hetgeen de histopathologische diagnostiek compliceert. Met name het onderscheid tussen een dieper dermaal deel van een melanoom versus een naevusrest gelegen onder een micro-invasief horizontaal groeiend melanoom is klinisch relevant omdat het van grote invloed is op de Breslow-dikte en dus op de prognose en het klinisch beleid. Als een mogelijke bijdrage om te komen tot de juiste diagnose wordt in hoofdstuk 4 een onderzoek beschreven waarin de expressie van de epidermale groei factor receptor (EGFR) in melanocytaire cellijnen en weefsels op eiwit en mRNA niveau werd nagegaan, teneinde de waarde van EGFR als een mogelijke progressie merker te bepalen. De EGFR expressie in zes humane melanoom cellijnen werd vergeleken, gebruikmakend van de monoclonale antilichamen 2E9, 425 en 225, terwijl vier immunocytochemische kleuringsprocedures werden toegepast. Een drie staps-immunoperoxidase methode met gebruikmaking van het 2E9 antilichaam was het meest gevoelig. De semikwantitatief ingeschatte aankleuringsintensiteiten correleerden goed met de kwantitatieve gegevens die verkregen waren middels een "multiple point ligand binding assay", die aantoonden dat EGFR expressie in de sterk metastaserende cellijnen MV3 en BLM tenminste 40 keer zo hoog was als in de cellijnen IF6, 530, M14 en Mel57, die na subcutane inspuiting bij naakte muizen niet of alleen sporadisch uitzaaien. De expressie op mRNA niveau volgens een Northern blot analyse was eveneens in overeenstemming met deze resultaten. Immunohistochemische kleuring van een grote reeks humane melanocytaire huidlaesies toonde een differentiële EGFR expressie in verschillende stadia van melanocytaire tumorprogressie: 19% van de naevocellulaire naevi van het gebruikelijke type toonde aankleuring van de melanocytaire cellen, terwijl dit 61% was voor de dysplastische naevi, 89% voor de primaire huidmelanomen en 91% voor de melanoom metastasen. Er werd geconcludeerd dat de EGFR expressie toeneemt bij melanocytaire tumorprogressie.

De hoofdstukken 5, 6 en 7 betreffen benaderingswijzen die gebruikt kunnen worden om bij de lastige differentiaal diagnose tussen het melanoom en een Spitz naevus, het aantal niet of moeilijk te classificeren gevallen te reduceren. Hiertoe werden DNA-ISH en allelotyperingsexperimenten uitgevoerd, gebruikmakend van routinematig verwerkte formaline-gefixeerde en paraffine-ingebedde weefsels.

In hoofdstuk 5 werd de mogelijkheid tot het toepassen van DNA-ISH op melanocytaire cellijnen en weefsels onderzocht, tegen de achtergrond van de mogelijkheid tot het bestuderen van genetische afwijkingen en heterogeniteit bij melanocytaire tumorprogressie. Bestudeerd werd de mogelijkheid om numerieke afwijkingen van de centromeer regio's van chromosoom 1 en 7 aan te tonen in intacte kernen van twee humane melanoomcellijnen met een verschillend metastaseringsgedrag in naakte muizen. Daarnaast werden paraffinecoupes gebruikt van materiaal afkomstig van tumoren verkregen door inspuiten van de cellijnen bij naakte muizen (subcutane tumoren en spontane longmetastasen). Ook werden paraffinecoupes van het oorspronkelijke primaire huidmelanoom (met een subepidermaal en een dermaal gedeelte) bestudeerd alsmede twee loco-regionale metastasen, waarvan er één het uitgangsmateriaal vormde voor de cellijnen. Voor wat betreft de cellijnen correleerde de ISH-analyse goed met gegevens gekregen middels ISH op metaphase preparaten. Verschillen tussen de cellijnen die niet konden worden gedetecteerd met flow-cytometrische of conventionele karyotyperingsanalyse waren een polyploide subpopulatie van tumorcellen en een extra copie van chromosoom 7 in alleen de metastaserende cellijn. De polyploide cellen konden ook gedetecteerd worden in paraffinecoupes van de corresponderende subcutane tumor en longmetastasen in de muizen. Beide gebieden in het primaire melanoom van de patiënt konden afzonderlijk geëvalueerd worden en toonden soortgelijke numerieke afwijkingen van de bestudeerde chromosoom regio's. Deze afwijkingen kwamen overeen met die in de beide metastasen. De resultaten toonden aan dat DNA-ISH gebruikt kan worden om genetische afwijkingen te visualiseren op het niveau van de enkele cel in melanocytaire kernen in hun natuurlijke context, hetgeen DNA-ISH een potentiële aanvullende techniek in de histopathologie van melanocytaire laesies maakt.

In hoofdstuk 6 is een studie beschreven naar de mogelijke implementatie van DNA-ISH bij de histologische differentiaal diagnostiek tussen Spitz naevus en melanoom. Hiertoe werden coupes gebruikt van vijftien typische Spitz naevi, vijftien typische nodulaire melanomen en vijf gevallen die oorspronkelijk diagnos-

tiseerd waren als Spitz naevus maar die later bleken te metastaseren en dus uiteindelijk geclassificeerd werden als melanoom. Deze gevallen, waarvan de weefsels routinematig verwerkt waren, werden geanalyseerd met behulp van DNA-ISH. Een microscopische, semikwantitatieve evaluatie toonde dat het aantal kernen met getalsmatige afwijkingen van de centromeer regio van chromosoom 1, hetgeen suggestief is voor aneuploidie, significant verschillend was in Spitz naevi en nodulaire melanomen. Het gemiddelde aantal afwijkende kernen bij hoge vergroting was 0.41, respectievelijk 4.01. Bij toepassing van deze resultaten op de oorspronkelijk incorrect geclassificeerde laesies konden drie van de vijf geïdentificeerd worden als melanoom. Deze resultaten wijzen erop dat de toepassing van DNA-ISH kan bijdragen aan het identificeren van een melanoom bij de differentiaal diagnose met een Spitz naevus.

In hoofdstuk 7 is een studie beschreven waarin ook routinematig behandelde weefsels werden gebruikt als uitgangsmateriaal om allelotyperingsexperimenten te doen op Spitz naevi en maligne melanomen teneinde oriënterend te onderzoeken of het verschijnsel van verlies van heterozygotie (LOH), als kenmerk voor de aanwezigheid van zogenaamde vroege of late genetische veranderingen, voor diagnostische doeleinden gebruikt kan worden. Hiertoe werden vijf microsatelliet merkers, te weten D3S1293, D6S271, D9S171, D10S187 en D18S70 gebruikt om LOH aan te tonen op de chromosoomregio's 3p, 6q, 9p, 10q en 18q. De technische procedure, waarbij gebruik gemaakt werd van geautomatiseerde op fluorescentie gebaseerde DNA-metingsapparatuur, werd voor deze toepassing geoptimaliseerd. De criteria voor de interpretatie van de uiteindelijke curves, welke werden geëvalueerd met het blote oog, ondersteund door het berekenen van de imbalans ratio tussen allelen, werden vastgesteld. Het totaal aantal testen dat evalueerbaar was voor wat betreft de aanwezigheid van LOH, bleek vergelijkbaar voor melanomen (29 testen) en Spitz naevi (27 testen). Het niet interpreteerbaar zijn van een test was te wijten aan homozygotie van de markers (21 testen), preferentiele PCR van een significant kleiner allel (5 testen) of aan onbekende factoren, die waarschijnlijk te maken hadden met slechte kwaliteit van het geisoleerde DNA (8 testen). Vijf van de negen melanomen toonden LOH van tenminste één locus, terwijl geen LOH gevonden werd in de negen onderzochte Spitz naevi. Bij de melanomen met LOH was altijd of de chromosoom 9 of de chromosoom 10 merker betrokken. Aangezien meerdere van de genoemde problemen kunnen worden verholpen door gebruik te maken van extra merkers, werd geconcludeerd dat het detecteren van LOH waardevol kan zijn als hulpmid-

del bij de differentiaal diagnose van melanoom versus Spitz naevus, hetgeen verder onderzoek rechtvaardigt.

Samenvattend zijn in dit proefschrift moleculaire en morfologische benaderingswijzen beschreven die van waarde kunnen zijn om specifieke problemen in de melanocytaire pathologie op te lossen. Sommige van de verkregen resultaten zijn inmiddels geïmplementeerd terwijl andere interessant zijn om verder te valideren voor een toekomstige toepassing in de diagnostische melanocytaire pathologie.

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# CURRICULUM VITAE

Peter Egbert Jan de Wit werd op 9 mei 1961 geboren te Bergen op Zoom, alwaar hij het gymnasium doorliep aan het R.K. Gymnasium Juvenaat H. Hart.
In 1979 begon hij met de studie Geneeskunde aan de Katholieke Universiteit Nijmegen. Na het doctoraal-examen in 1984 volgde hij co-assistentschappen in het Academisch Ziekenhuis Nijmegen St Radboud en in geaffilieerde ziekenhuizen, gevolgd door een klinisch onderzoeksjaar op de afdelingen Dermatologie en Kinderoncologie van het Academisch Ziekenhuis Nijmegen St Radboud.
Na het artsexamen eind 1987 vervulde hij de militaire dienstplicht als artsassistent interne geneeskunde in het Militair Hospitaal A. Matthijsen te Utrecht.
In 1989 begon hij als wetenschappelijk onderzoeker bij de afdeling Pathologie, Academisch Ziekenhuis Nijmegen St Radboud, waarbij de basis gelegd werd voor dit proefschrift. Daarnaast werd op dezelfde afdeling in 1992 aangevangen met de opleiding tot patholoog.
In de zomer van 1997 wordt de auteur van dit proefschrift geregistreerd als klinisch patholoog.

Stellingen

behorende bij het proefschrift

**Molecular and morphological approaches in diagnostic melanocytic pathology**

Peter de Wit, 5 juni 1997

1. Bij het stellen van de histopathologische diagnose maligne melanoom op de kinderleeftijd moet grote terughoudendheid betracht worden.
(dit proefschrift; Spatz et al., Int J Cancer 1996; 68: 317-324)

2. De histopathologisch bepaalde grens tussen een naevus naevocellularis van het gebruikelijke type enerzijds en een dysplastische naevus anderzijds kan het beste getrokken worden op basis van de volgende kenmerken: a. sterk toegenomen grensvlakactiviteit; b. onregelmatige nesten; c. grote kernen; d. lymfohistiocytair infiltraat.
(dit proefschrift)

3. Immunohistochemische en biochemische analyses zijn complementair bij het onderzoek van eiwitten in weefsels.

4. DNA-In Situ Hybridisatie en genetische analyse van verlies van heterozygotie kunnen bijdragen tot het diagnostiseren van een maligne melanoom door de patholoog.
(dit proefschrift)

5. Moleculaire diagnostiek en prognostiek in de oncologie vergt bundeling van krachten vanuit meerdere disciplines, waaronder zeker de pathologie.

6. Kinderen die een leukemie en de behandeling daarvoor overleefd hebben moeten gecontroleerd worden op (pre-)maligne melanocytaire afwijkingen van de huid.
(De Wit et al., Med Pediatric Oncol 1990; 18: 336-338; Curtis et al, NEJM 1997, 336: 897-904)

7. Bij een krimpend budget krijgt het woord kwaliteitszorg een accentverschuiving van verzorging naar bezorgdheid.

8. Zwijgen is alleen dan goud indien deze tijd gebruikt wordt om na te denken over wat men moet gaan zeggen.

9. De fixatie van veel pathologen op fixatie-technieken is niet pathologisch.

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